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GENETICS OF RESISTANCE TO RADIATION IN *ESCHERICHIA COLI*[1,2]

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INTRODUCTION

A MUTATION in *Escherichia coli*, leading to heightened resistance to ultraviolet radiation and x-rays, was described in an earlier publication (WITKIN 1946). Evidence was presented that this mutation occurs spontaneously in cultures of the parent strain. In the present paper, a more complete account of these preliminary investigations will be given, as well as additional experiments to clarify the genetic basis of resistance to radiation.

This mutation is one of a number of bacterial variations which have been found, in recent years, to exhibit striking similarities to mutational changes in higher plants and animals. Since the standard techniques of Mendelian genetics have not been applicable to bacteria, these analogies afford the only available evidence that bacterial heredity is gene-controlled. Among the similarities between variations in bacteria and mutations in higher organisms which are most suggestive of a common underlying genetic basis are the following: (1) Many variations in morphological and physiological characters occurring in cultures of bacteria are transmissible, unchanged, through numerous generations, and are therefore considered to be stable and heritable (see, for example, MASSINI 1907, LEWIS 1933, SEVERENS and TANNER 1945). (2) The spontaneous origin of certain stable and heritable variations, independent of the specific treatments used to detect them, has been proven in a few cases by means of special techniques (LURIA and DELBRÜCK 1943, DEMEREC 1945, RYAN, SCHNEIDER and BALLENTINE, 1946). (3) Different characters within a strain may vary independently of one another (REED 1937, AUDUREAU 1942). In one instance, the rate of mutation to a particular variant type was found to be the same for strains differing by one or more other mutational steps (DEMEREC and FANO 1945). (4) Physical and chemical agents known to be effective in increasing the rates of mutations in higher organisms have similar effects on bacteria (for the effects of x-rays, see HABERMAN and ELLSWORTH 1940, GRAY and TATUM 1944; for x-rays and ultraviolet, DEMEREC 1946; for mustard gas, TATUM 1946). The mutations induced by these agents

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[2] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, COLUMBIA UNIVERSITY.

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in bacteria, as in higher organisms, seem to be random and nonspecific. (5) Certain variations in bacteria leading to altered growth requirements and synthetic abilities (ROEPKE, LIBBY and SMALL 1944, GRAY and TATUM 1944) are close analogues of the biochemical mutations in Neurospora, a sexually reproducing microorganism in which the existence of gene-controlled heredity has been established with certainty.

Bacterial variations which meet the tests of stability and heritability, of spontaneous origin at more or less constant rates, of responsiveness to known mutation-inducing agents, etc., are likely to be the most promising materials for the investigation of bacterial heredity. Mutations involving resistance to destructive agents, such as bacteriophage (LURIA and DELBRÜCK 1943), penicillin (DEMEREC 1945) or radiation, are especially suitable for genetic analysis, since resistant mutants can be detected easily in cultures of sensitive bacteria.

Apart from its amenability to investigation, resistance to radiation may be of particular significance for genetics. The fact that radiobiological results obtained in the study of the bactericidal effect of radiation parallel those obtained for the production of mutations in *Drosophila* (see LEA 1946) suggests that the killing of bacteria by radiation may be mediated through an effect on some center of genetic importance. This possibility is also supported by the similarity between the absorption spectrum of nucleic acids and the efficiency spectrum for the killing of bacteria by ultraviolet radiation (GATES 1928). A mutation which alters the sensitivity of bacteria to radiation, therefore, is likely to effect a basic change in the genetic economy of the organism.

Since the early researches of DOWNES and BLUNT (1877), the bactericidal action of radiation has been a prominent subject for radiobiological investigation. Differences in sensitivity within a strain have often been reported, but these have usually been found to depend upon transient physiological factors, such as the age of the culture. Inherited differences in sensitivity to ultraviolet radiation among clones of *E. coli* were described by RENTSCHLER, NAGY and MOUROMSEFF (1941), and HOLLAENDER (1942) noted that one in a million bacteria in a population of *E. coli* could survive very high doses of radiation.

It was our purpose in these experiments to determine the extent to which resistance to radiation may be considered a true mutational change, and to establish its genetic basis insofar as possible.

MATERIAL AND METHODS

Strain *B* of *Escherichia coli* and mutants derived from this strain were used throughout these experiments. The original culture, obtained from DR. M. DEMEREC, was diluted and plated on agar so as to give a few well-separated colonies after incubation. A stock culture was prepared by inoculating one of these colonies on a nutrient agar slant. The stock was carried by subculturing the slant every two months. Stocks of mutant strains were carried in the same way. Cultures to be used for comparable experiments were inoculated with samples from the same slant.

The media used, unless otherwise specified, were Difco nutrient agar and

Difco nutrient broth. The synthetic medium used in certain experiments consisted of a buffered solution of inorganic salts with ammonium chloride as the source of nitrogen, and glucose as the sole carbon source.

The source of ultraviolet radiation was a General Electric germicidal lamp, of the low-pressure mercury vapor type, emitting ultraviolet radiation about 80 percent of which is of wave length 2537 Å. The output was stabilized by an automatic regulator, and the intensity at a distance of 92 cm from the source was about 4.2 ergs per square millimeter. The lamp was calibrated in absolute units by DR. R. LATARJET.

In most of the experiments, bacteria to be irradiated with ultraviolet were taken from 24-hour broth cultures, and diluted quantitatively in broth. Measured samples were spread evenly on the surface of nutrient agar Petri dishes with a sterile glass rod. The plates were then exposed to the radiation, and colony counts were made after 24 hours of incubation. Survival was measured by comparison with nonirradiated control plates. Special methods used in certain instances will be described in connection with the specific experiments.

Irradiation with x-rays was conducted at MEMORIAL HOSPITAL in New York City, through the courtesy of MR. L. D. MARINELLI, and with the assistance of MISS E. FOCHT. The tube was operated at 180 kv., and the intensity was 2050 roentgens per minute at the distance used. The radiation was administered without special filtration, but before reaching the bacteria the rays passed through the glass walls of the x-ray tube and of the tube containing the bacteria.

Bacteria to be irradiated with x-rays were taken from undiluted 24-hour aerated broth cultures, and were exposed in small, thin-walled glass tubes. Measured dilutions were made after irradiation, and were plated out. Colony counts were made after incubation, and were compared with nonirradiated controls.

Assays to determine the titre of liquid cultures were made by the usual method of plating measured dilutions and making colony counts. Similar assay methods were used in the preparation of growth curves, starting the cultures with measured inocula, and sampling at intervals over a 24-hour period.

EXPERIMENTAL

Isolation of the Resistant Mutant

From a culture of strain *B*, a sample of about 5×10^4 bacteria was taken, and irradiated with an ultraviolet dose of 1000 ergs/mm^2. Four colonies were found to have developed after incubation, indicating that only four bacteria had survived the treatment. Each of the colonies was inoculated into a tube of broth, and, at the same time, four control cultures were started by inoculating into broth single colonies from a nonirradiated plate. The sensitivity to ultraviolet of the two sets of cultures was compared, by irradiating samples from each of the cultures with two test doses. The results are shown in table 1. The cultures derived from the four survivors of the original irradiation were characterized by considerably greater resistance to ultraviolet than corresponding culture from control colonies.

One of these four resistant cultures (no. 1) was selected to serve as a stock resistant strain for further study, and was established on an agar slant. This strain was designated as strain *B/r* (*B* resistant to radiation).

Some Properties of the Resistant Strain

(1) Stability of Strain *B/r*

The resistant strain *B/r* has been carried through over 50 successive subcultures in broth, and for a period of over two years on agar, with frequent

TABLE 1

Sensitivity to ultraviolet of bacteria surviving irradiation with a high dose of ultraviolet.

Origin of control cultures: single colonies from a non-irradiated plate seeded with bacteria from strain *B*. Origin of experimental cultures: single-colony survivors from plates seeded with bacteria from strain *B*, and irradiated with a dose of 1000 ergs/mm².

CULTURE	TREATMENT: 90 ergs/mm²		TREATMENT: 500 ergs/mm²	
	CELLS PER SAMPLE	SURVIVAL PERCENT	CELLS PER SAMPLE	SURVIVAL PERCENT
Control—1	520	3.5	1040	0.4
Control—2	481	5.4	962	0.5
Control—3	456	2.9	912	0.9
Control—4	509	5.9	1018	0.2
Experimental—1	455	92.9	890	37.7
Experimental—2	520	97.5	1040	40.0
Experimental—3	490	98.3	980	43.2
Experimental—4	512	92.3	1024	38.1

single-colony isolations. No change has been observed in sensitivity to ultraviolet, or in other characteristics studied. Resistance to ultraviolet may, therefore, be considered a stable, heritable character.

(2) Sensitivity of Strain *B/r* to Ultraviolet

The curves of survival of strains *B* and *B/r* as a function of ultraviolet dose are shown in Figure 1. Two sets of curves are represented, one set (A) obtained when the irradiation is administered to bacteria spread on the surface of agar medium, and the other set (B) when the bacteria are irradiated in liquid suspension. For purposes of comparing the sensitivity of the parent and mutant strains, the curves made under one or the other of these conditions should be examined. In addition to the difference in level of sensitivity, there is a marked difference in the shape of the survival curves of the parent and mutant strains. For this reason, a numerical coefficient to express the difference in sensitivity between the two strains would be meaningless.

The significance of the difference in sensitivity observed when irradiation is carried out on solid or in liquid medium will be considered in the Discussion.

(3) Sensitivity of Strain *B/r* to X-rays

The survival curves of strains *B* and *B/r* with x-rays are shown in figure 2. The mutant strain exhibits relative resistance to x-rays, as well as to ultraviolet. The shape of the x-ray survival curve of strain *B/r* is linear, in contrast to the sigmoid curve obtained for this strain with ultraviolet.

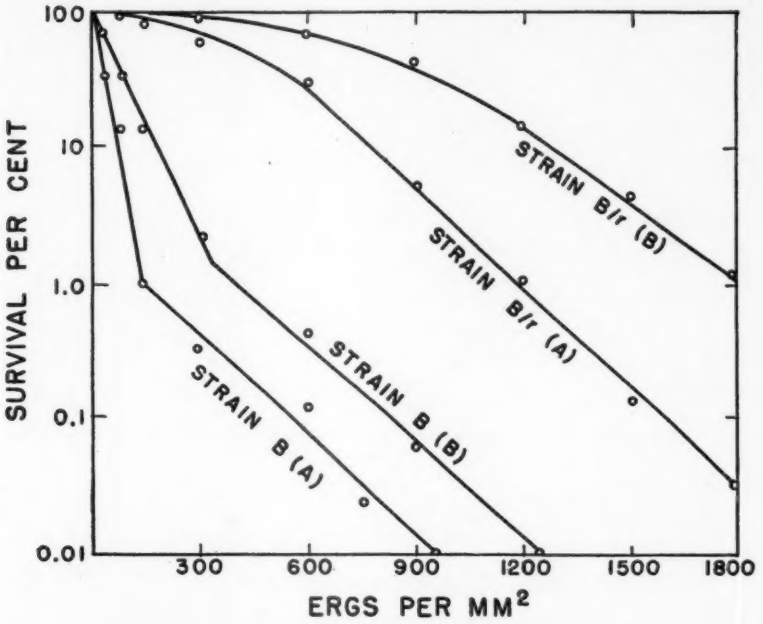

FIGURE 1.—Sensitivity of strains *B* and *B/r* to ultraviolet radiation.
(A) bacteria irradiated on surface of solid medium.
(B) bacteria irradiated in liquid suspension.

The survival curves for the parent strain *B* with both ultraviolet and x-rays show a change of slope at about one percent survival. The change in rate of death at this point cannot be accounted for by the presence of resistant mutants in the normal samples.

(4) Growth Characteristics of Strain *B/r*

The growth rates of strains *B* and *B/r* in broth at 37°C were compared. Differences in growth rates between the two strains are confined to the lag

phase before the onset of logarithmic growth, and to the final titre. The lag phase of the resistant strain, defined as the time required for the doubling of the initial inoculum, was found to be one hour, as compared with ninety minutes for the parent strain. The generation time (19 minutes) was the

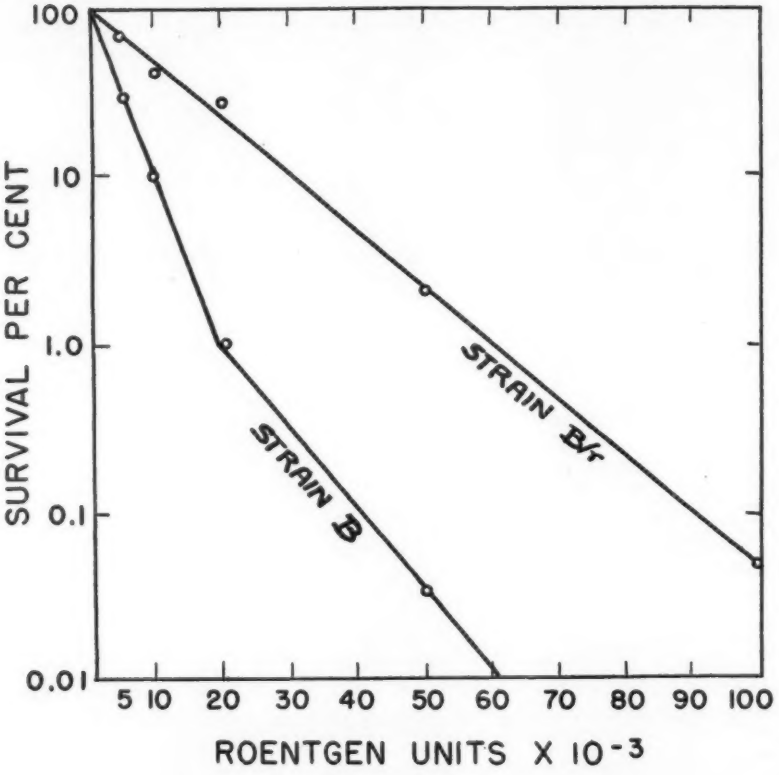

FIGURE 2.—Sensitivity of strains *B* and *B/r* to x-rays.

same for both strains, but the final titre of resistant cultures is usually slightly lower than that of sensitive cultures.

Both strains are able to grow luxuriantly in synthetic medium without added growth factors.

(5) Behavior of Individual Cells after Irradiation with Ultraviolet

Another difference between sensitive and resistant bacteria was observed by examining under the microscope bacteria irradiated with low doses of ultraviolet. It is known (GATES 1933) that ultraviolet radiation, in low doses, is

one of a number of agents which exert a specific inhibiting effect on the division of bacterial cells, without affecting growth in length, resulting in the production of long filaments.

Samples from the parent strain (B) were irradiated with a dose of 50 ergs per mm², incubated, and examined at intervals on the surface of the agar plate under high-power magnification. Almost every cell was seen to elongate into a filament, reaching a length of 50–100μ within three to four hours after treatment. No evidence of division was observed until after four hours of incubation.

Samples from the resistant strain (B/r) were irradiated with the same dose, incubated, and examined in the same way. Almost every cell began to divide

TABLE 2

Sensitivity of strains B and B/r to penicillin and sodium sulfathiazole.

	CONCENTRATION OF PENICILLIN—UNITS/ML			CONCENTRATION OF SODIUM SULFATHIAZOLE—MOLES/ML		
	20	40	60	1×10^{-6}	2.5×10^{-6}	5×10^{-6}
Growth of strain B	+	−	−	+	−	−
Growth of strain B/r	++	++	−	++	++	+

++ = Full turbidity after 72 hours.
+ = Less than full turbidity after 72 hours.
− = No visible turbidity after 72 hours.

within one hour, the normal lag, and formed a microcolony of 50–100 normal-sized cells within three to four hours.

Strain B/r thus exhibits resistance not only to the lethal action of ultraviolet radiation, but also to its inhibitory effect on cell division. The technical usefulness of this difference for the genetic analysis of the mutation will be apparent in a later section.

(6) Sensivitity of Strain B/r to Penicillin and Sulfathiazole

Penicillin and sulfathiazole are antibacterial agents which are also known to cause inhibition of cell division, with production of elongated filaments, when administered to sensitive cells in low concentrations. The possibility was considered that the resistance of strain B/r to radiation might extend to other agents exerting a specific division-inhibiting effect. Experiments were conducted to compare the sensitivity of strains B and B/r to penicillin and sodium sulfathiazole. Table 2 gives the results, which indicate that the mutant B/r is relatively resistant to these agents, as well as to ultraviolet radiation and x-rays.

A Technique for the Quantitative Detection of Radiation-Resistant Bacteria in Samples from Sensitive Cultures

The genetic analysis of mutations in bacteria is dependent upon selective procedures which permit the determination of the number of mutants present in samples from parent cultures. Since the resistance to radiation characterizing strain *B/r* is relative, rather than absolute, no such procedure was readily available.

If spontaneous mutation is wholly or partially responsible for the change in sensitivity, a sensitive culture must contain, prior to irradiation, a certain proportion of resistant cells. The survival curves for sensitive and resistant bacteria show that a dose sufficiently high to eliminate all or most of the sensitive bacteria will, at the same time, eliminate most of the resistant cells. Treatment with a high dose of ultraviolet will thus permit the recovery of only a small fraction of the resistant bacteria initially present in the sample.

A special method, designated as the double-irradiation technique, was developed, whereby all the resistant bacteria in a normal sample could be detected selectively. This method was utilized in many of the experiments to be reported below, and will therefore be described in some detail.

The technique makes use of the difference, described above, in the behavior of individual bacteria of the parent and mutant strains after irradiation with low doses of ultraviolet. It will be remembered that sensitive bacteria, irradiated with a low dose, form undivided long filaments, 50–100μ in length, after three to four hours of incubation. Resistant bacteria, irradiated with the same dose, form microcolonies of 50–100 cells of normal size after the same period of incubation. The dose employed (50 ergs per mm^2) kills all but 10 percent of the sensitive bacteria, but permits the survival of 100 percent of the resistant mutants.

Measured samples from cultures of strain *B*, strain *B/r* and artificially prepared mixtures of the two strains were spread on agar plates, irradiated with a dose of 50 ergs per mm^2 and incubated for three hours at 37°C. At the end of the incubation period, each of the plates was irradiated a second time, with a dose of 700 ergs per mm^2. It will be seen from the survival curve (figure 1) for strain *B/r* that this dose allows the survival of 20 percent of the resistant bacteria. Therefore, about 10–20 cells in each resistant microcolony of 50–100 cells (formed during the incubation period between the first and second irradiations) should survive the second treatment. If at least *one* cell in each resistant microcolony survives the second irradiation, a visible colony should form, after suitable incubation, for every resistant bacterium originally present in the sample.

On the other hand, the second dose of 700 ergs per mm^2 permits the survival of only 0.05 percent of the sensitive bacteria. Since 90 percent of the sensitive cells are eliminated by the first irradiation, the overall survival of sensitive bacteria after the two treatments should be about .005 percent. If the original sample is no larger than 2×10^4 cells, therefore, it should be possible to eliminate *all* the sensitive bacteria by the same treatment which insures 100 per-

cent survival of resistant bacteria. This will be possible, of course, only if the filamentous cells produced by the first irradiation behave like single bacteria rather than like chains of bacteria in their sensitivity to radiation. LEA, HAINES and COULSON (1937) found that this was true of long forms produced by gamma rays.

Table 3 gives the results of an experiment to determine the validity of this technique. When samples from cultures of strain *B* were given the double-

TABLE 3

Reliability of double-irradiation technique for selective recovery of radiation-resistant bacteria in mixture with sensitive bacteria.

Double-irradiation technique: first dose of 50 ergs/mm² followed by three hours of incubation and second dose of 700 ergs/mm².

CULTURE	SAMPLE	INITIAL NUMBER OF CELLS PER SAMPLE		COLONY COUNT AFTER TREATMENT AND INCUBATION	
		B	*B/r*	SENSITIVE	RESISTANT
Normal (*B*)	1			0	1
	2			0	0
	3			0	0
	4			0	1
		Av. 11,200	—	—	—
Resistant (*B/r*)	1			0	114
	2			0	91
	3			0	120
	4			0	130
		—	Av. 111±9.0	—	Av. 113.7±16.5
Mixture (*B*+*B/r*)	1			0	94
	2			0	112
	3			0	106
	4			0	96
		Av. 9,400	Av. 106±8.5	—	Av. 102.0±8.5

irradiation treatment, the only survivors obtained proved to be resistant mutants. When samples from cultures of the resistant strain *B/r* were treated, the number of colonies obtained corresponded to the number of bacteria originally plated. When an artificial mixture of the two strains was treated, only resistant colonies were obtained, and these corresponded in number to the proportion of resistant bacteria in the mixed sample originally plated. Every colony was characterized with respect to sensitivity or resistance by a simple and rapid diagnostic test. An inoculum from each colony to be tested was suspended in a small volume of broth, and a loopful of the suspension was drawn across a small segment of an agar plate. About twenty colonies could be

tested on a single plate. The plates were irradiated with a dose of 50 ergs per mm^2, and incubated at 37°C for three hours. Each segment was then examined under the microscope. If the colony tested was composed of sensitive bacteria, long thread-like filaments were observed; if the colony consisted of resistant cells, microcolonies of 50–100 cells of normal size were seen. The difference between the long sensitive filaments and the compact resistant microcolonies is so striking that the diagnosis can be made in a few seconds, under low power magnification.

The results of the experiment reported in table 3 indicate that the double-irradiation technique is effective in providing an absolute selective procedure for the detection of radiation-resistant mutants.

The technique can be modified to determine the number of resistant mutants in samples as large as 10^7, by extending the time of incubation between irradiations up to five hours, and increasing the second dose up to 1800 ergs per mm^2. When large samples are used, however, sensitive bacteria occasionally survive, probably because of screening by the dense network of elongated cells which develops after the first irradiation. When samples of about 10^6 bacteria or more are used, the diagnosis of each surviving colony for sensitivity or resistance becomes a necessary part of the procedure.

Origin of the Change from Sensitivity to Resistance

The proof of the spontaneous origin of hereditary variations in bacteria at more or less constant rates under given conditions, is an important means of establishing the underlying similarity of these changes to mutations in higher organisms. In the case of radiation-resistance, this problem is of particular interest, since ultraviolet radiation and x-rays are known to be effective mutation-inducing agents.

The following possible modes of origin of the change from sensitivity to resistance may be postulated: (1) The change may be induced by the radiation in a certain number of cells in an initially homogeneous population, or (2) the change may be a spontaneously occurring mutation, and prior to the treatment the culture contains a certain number of resistant mutants. In this case (a) the radiation may act merely as a selective agent, or (b) it may act as a selective agent, but also as an inducing agent, increasing the rate of mutation to resistance.

The method used to test these hypotheses was developed by LURIA and DELBRÜCK (1943), and was applied by them and by DEMEREC (1945) to the study of bacterial mutations. The method involves the following considerations:

If the change is entirely induced by the radiation, the number of resistant bacteria obtained from a sample will depend upon the probability that an induced change will occur in any bacterium. This probability should be the same for all bacteria under similar physiological conditions. Therefore, the number of resistant bacteria in samples from a series of similar, independent cultures should show fluctuations no greater than those shown by the number of resistants in a series of samples from a single culture. These fluctuations

TABLE 4

Number of resistant bacteria in samples from independent cultures, and in samples from single cultures.

Number of resistant bacteria determined by double-irradiation technique: first dose of 50 ergs/mm² followed by 5 hours of incubation, and second dose of 1500 ergs/mm².

	SAMPLES FROM INDEPENDENT CULTURES				SAMPLES FROM SINGLE CULTURES		
EXPT. NO.	1	2	3		1	2	3
AV. NO. CELLS PER SAMPLE	1×10^6	1.1×10^6	9.5×10^5		9.7×10^5	1×10^6	1.2×10^6
SAMPLE NO.				SAMPLE NO.			
1	0	4	5	1	8	0	13
2	12	8	13	2	10	1	9
3	8	15	61	3	5	3	11
4	8	12	10	4	9	1	14
5	19	0	1	5	7	0	9
6	98	14	12	6	9	0	8
7	7	1	2	7	15	2	12
8	5	76	8	8	8	1	13
9	14	11	0	9	6	4	7
10	24	42	13	10	16	1	15
11	—	—	9	11	—	—	8
12	—	—	7	12	—	—	8
13	—	—	18	13	—	—	14
14	—	—	0	14	—	—	9
15	—	—	11	15	—	—	13
16	—	—	8	16	—	—	6
17	—	—	116	17	—	—	11
18	—	—	12	18	—	—	7
19	—	—	10	19	—	—	10
20	—	—	6	20	—	—	18
Average	19.5	18.3	16.1		9.3	1.3	10.8
Variance	764.7	574.7	1509.8		12.9	1.8	6.9
χ^2	373.3	279.6	844.0		12.5	11.6	12.2
P	—	—	—		0.1–1.2	0.2–0.3	0.8–0.9

should be due only to sampling error, and in both cases the distribution of the number of resistant bacteria should constitute a Poisson series, with the variance approximately equal to the mean.

If the change is a spontaneous mutation, the number of resistant bacteria obtained from a given sample depends upon (1) the probability that any bacterium will mutate during its lifetime, and (2) the time of occurrence of mutations during the growth of the culture, since all bacteria descended from mutated cells will be resistant. In this case, the number of resistants in samples

from a series of similar, independent cultures should show large fluctuations (see LURIA and DELBRÜCK 1943) and the variance should be significantly higher than the mean.

Table 4 gives results of experiments to determine the number of resistant bacteria in samples from a series of similar, independent cultures, and in samples from a single culture. Every culture was started with an inoculum of about 20 cells from the sensitive strain, in a volume of 1 ml. of broth. The maximum difference in final titre of the cultures in any series was 12 percent.

In all three experiments, the number of resistant bacteria in samples from a single culture shows fluctuations satisfactorily accounted for by sampling errors. This indicates that the method of plating and irradiating does not introduce fluctuations beyond those expected on the basis of sampling.

The number of resistant bacteria in samples from a series of independent cultures, in all three experiments, shows fluctuations much greater than can be accounted for by sampling errors. The variance is significantly higher than the mean in every case, and the fluctuations are of the type to be expected according to the hypothesis of spontaneous mutation.

Although these experiments confirm the hypothesis of spontaneous origin of resistance to radiation, the possibility remains that the treatment used to detect these mutants induces the change in an additional number of sensitive bacteria. Under the conditions of the double-irradiation technique, however, there is only one class of induced mutation that could possibly appear, namely, those which become phenotypically resistant with no delay. Since no phenotypically sensitive cell can survive the treatment, those sensitive bacteria in which a mutation is induced, but does not take effect immediately, will have no chance to survive. DEMEREC (1946) has shown that, in the same strain of *E. coli*. the majority of ultraviolet-induced mutations to bacteriophage-resistance take effect only after cell division has occurred.

Direct experimental evidence was obtained which further weakens the likelihood that any significant number of the resistant mutants detected by the double-irradiation technique is due to induction. It has been mentioned that the doses used in this technique, depending upon the size of the sample, can vary from 700 to 1800 ergs per mm^2. An experiment was conducted to determine the number of resistant bacteria in several samples from the same culture, using doses throughout this range. The proportion of mutants in all of these samples was the same within the limits of sampling error. If a significant fraction of these mutants arises by induction, their number should vary with the dose.

An Estimate of the Mutation Rate

It is possible to estimate the rate of bacterial mutations from experiments of the type described above by solving the following equation: $r = aN_t ln(CaN_t)$ (for derivation see LURIA and DELBRÜCK 1943), where r is the experimental average of the number of mutants in a series of similar cultures, N_t is the number of bacteria at the time of observation, C is the number of cultures and a is the mutation rate.

LURIA and DELBRÜCK have plotted a series of curves relating the observed values of r to aN_t for various values of C. The estimated mutation rate for radiation-resistance, obtained from these curves, is about 10^{-5} mutations per bacterium per generation.

Induction of the Mutation to Radiation-resistance by Ultraviolet

It has been demonstrated that resistance to radiation is due to a spontaneous mutation, and that, under the conditions of the double-irradiation technique, the mutants are passively selected by the radiation. Experiments were conducted to determine whether, under suitable conditions, the mutation could be induced by radiation.

TABLE 5

Induction of mutations to radiation-resistance by treatment with a high dose of ultraviolet.

Culture used: 24-hour aerated culture of strain *B* in nutrient broth.
Spontaneous frequency of radiation-resistant mutants, determined by double-irradiation
Treatment: Experiment no. 1—.001%
Experiment no. 2—.005%

	INITIAL NO. OF BACTERIA	DOSE ADMINISTERED	AV. NO. OF COLONIES PER PLATE	TOTAL NO. OF COLONIES TESTED FOR RES. TO U.V.	NO. OF COLONIES RES. TO U.V.	% OF RES. MUTANTS AMONG SURVIVORS
Experiment no. 1						
Control	2×10^2	None	2×10^3	500	None	—
Experimental	2.4×10^8	3800 ergs/mm²	26	434	21	4.9
Experiment no. 2						
Control	1.7×10^2	None	1.7×10^3	365	None	—
Experimental	2.0×10^8	3800 ergs/mm²	30	450	15	3.3

The results of these investigations are shown in table 5. Samples taken from a 24-hour aerated culture of strain *B* were irradiated with a dose of about 3800 ergs per mm². Nonirradiated control plates were spread with suitably diluted samples from the same culture. After incubation, several hundred colonies from the treated plates, and a similar number from the control plates, were isolated and diagnosed for resistance to ultraviolet by the usual method of microscopic examination. A determination of the spontaneous frequency of mutants in the original culture was made by the double-irradiation method. The frequency of radiation-resistant mutants among the survivors of the high-dosage irradiation was found to be 4.9 percent in one experiment, and 3.3 percent in the other. No mutants were found among the control colonies in either experiment. The spontaneous frequency of mutants in the original

culture was about .001 percent in one experiment, and .005 percent in the other.

Although the values of 4.9 percent and 3.3 percent may be slightly exaggerated by the differential survival of resistant mutants, these experiments indicate that the rate of mutation to radiation-resistance can be increased considerably by high doses of ultraviolet.

The Number of Independent Mutations Resulting in Resistance to Radiation

In the foregoing section, resistance to radiation has been discussed as if a single mutation were responsible for this character. Techniques comparable to the analysis of breeding ratios and gene localization are not available to investigators of bacterial heredity, and it is necessary to approach this question with indirect and less satisfactory methods. A certain amount of information can be gained if the mutant differs from the parent strain in a number of independently demonstrable phenotypic characters. If a series of unrelated strains of the mutant is isolated, and tested with respect to all of these properties, it may be possible to divide them into a number of distinct classes, differentiated on the basis of one or more secondary characteristics. In favorable instances, the number of classes differentiated in this way may be shown to correspond to a minimum number of separate mutations, each capable of producing the primary phenotypic character, which may or may not be associated with the various secondary changes.

An analysis of this type was made of a group of 50 unrelated strains of the radiation-resistant mutant. Each of the strains was isolated from a different culture of strain *B*, started with an inoculum of about 20 bacteria. A sample from each of the cultures was subjected to the double-irradiation treatment, and one resistant colony from each plate was selected and established on a agar slant.

The properties chosen for analysis were those whereby the original mutant strain *B/r* was known to differ sharply from the parent strain: sensitivity to ultraviolet radiation, x-rays, penicillin and sulfathiazole; lag phase in broth at 37°C, and filament formation after exposure to an ultraviolet dose of 50 ergs/mm^2. All 50 strains were characterized with respect to each of these properties, except that only ten strains were tested for resistance to x-rays. The results are summarized in table 6. The 50 unrelated strains were found to be identical with respect to all of the properties examined, except resistance to penicillin and sulfathiazole. Four classes of mutants were differentiated on the basis of these characters: 21 strains were resistant to both of these agents; eight were resistant to penicillin, but not to sulfathiazole; five were resistant to sulfathiazole, but not to penicillin, and 16 were resistant to neither of these agents.

These results constitute indirect evidence that at least four separate mutations to radiation-resistance are possible, distinguishable by the presence or absence of associated resistance to penicillin and sulfathiazole. This conclusion, of course, would not be justified if the resistance to penicillin and sulfathiazole associated with radiation-resistance is due to coincidence of independent muta-

tions. The relative frequencies of the four classes of mutants militate strongly against this possibility. If coincidence of three independent mutations were necessary to produce simultaneous resistance to radiation, sulfathiazole and penicillin, the rarest class should be the triple mutants. Actually, the most frequent class of mutants is that exhibiting resistance of all three agents.

Radiation-resistance as a "Modifier" of Penicillin-resistance

The experiments described in this section were designed to clarify the resistance to penicillin characterizing many radiation-resistant strains.

Curves of survival of strains *B* and *B/r* as a function of penicillin concentra-

TABLE 6

Analysis of properties of 50 unrelated radiation-resistant strains.

PROPERTIES EXAMINED	LAG PHASE IN BROTH AT 30°C	FORMATION OF FILAMENTS 3 HRS. AFTER IRRADIATION WITH 50 ERGS/MM²	RESISTANCE TO ULTRAVIOLET: DOSE AT WHICH 50% SURVIVE	RESISTANCE TO X-RAYS: DOSE AT WHICH 50% SURVIVE	RESISTANCE TO PENICILLIN: GROWTH IN 40 U/ML	RESISTANCE TO SODIUM SULFATHIAZOLE: GROWTH IN 2.5×10^{-4} MOLES/ML
Strain *B*	1 hr., 20 min.	Filaments 50–100μ in length	About 30 ergs/mm²	About 5 r	No growth	No growth
Strain *B/r*	1 hr.	No filaments	About 450 ergs/mm²	About 15 r	Full growth	Full growth
Unrelated radiation—resistant strains:						
1–10	1 hr.	No filaments	About 450 ergs/mm²	About 15 r	Full growth	Full growth
11–21	1 hr.	No filaments	About 450 ergs/mm²	—	Full growth	Full growth
22–29	1 hr.	No filaments	About 450 ergs/mm²	—	Full growth	No growth
30–34	1 hr.	No filaments	About 450 ergs/mm²	—	No growth	Full growth
35–50	1 hr.	No filaments	About 450 ergs/mm²	—	No growth	No growth

tion were made, by plating measured samples of bacteria on penicillin agar. These curves are shown in Figure 3.

Samples from cultures of strains *B* and *B/r* were then plated on agar containing concentrations of penicillin ranging from 30 to 60 units per ml, and the plates were incubated for 48 hours. Forty-five colonies from the *B* series, and 50 colonies from the *B/r* series were isolated and inoculated into broth containing no penicillin. Each of the cultures was then tested roughly for resistance to penicillin, by inoculation into broth containing three critical concentrations of penicillin. Twenty-two of the cultures in the *B* series were able to grow in higher concentrations of penicillin than strain *B*, and were therefore considered to be penicillin-resistant mutants of this strain. They were designated as *B/p*. Seventeen of the cultures from the *B/r* series were able to grow in higher concentrations of penicillin than strain *B/r*. They were

considered to be penicillin-resistant mutants of strain *B/r*, and were designated as *B/r/p*.

Curves of survival of 15 *B/p* mutants and 15 *B/r/p* mutants as a function of penicillin concentration were made. These curves are shown in figure 3,

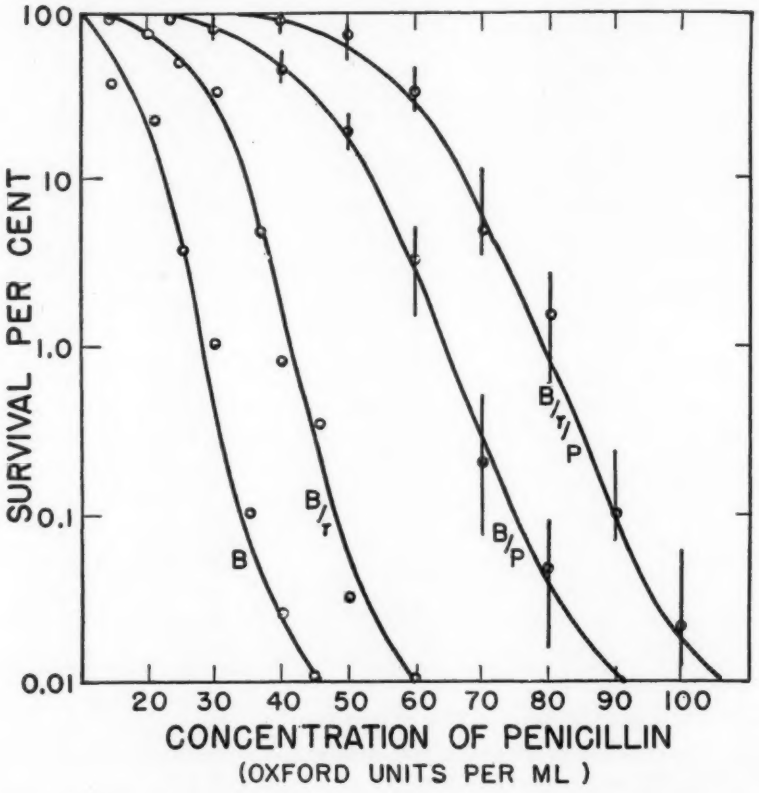

FIGURE 3.—Sensitivity of strains *B*, *B/r*, *B/p* and *B/r/p* to penicillin. Vertical lines drawn through points indicate limits of variability among 15 strains of *B/p* and 15 strains of *B/r/p*.

with the curves for each of the parent strains. The points represent average values for 15 strains, and the limits of variability are indicated by vertical lines drawn through each of the points.

When the four curves in figure 3 are compared, the following observations emerge: (1) Penicillin-resistant mutants obtained from strain *B*(*B/p*) are characterized by a higher level of resistance to penicillin than that shown by strain *B/r*. This observation supports the view that coincidence of independent

mutations is not responsible for the association of resistance to penicillin and to radiation found in strain *B/r*. (2) Penicillin-resistant mutants obtained from strain *B/r* (*B/r/p*) are more resistant to penicillin than mutants obtained from strain *B* by the same treatment. The survival curves for *B/p* and *B/r/p* are similar, but the resistance of the latter to penicillin is shifted to a higher level by an amount which corresponds approximately to the initial difference between strains *B* and *B/r*. Thus, the mutation to radiation-resistance, when it results in resistance to penicillin as well, acts as a "modifier" of an independent mutation to penicillin-resistance, adding quantitatively to its effect.

An additional observation was made which lends support to these conclusions. Ultraviolet-resistant mutants were isolated from a *B/p* mutant strain by the double-irradiation treatment, and survival curves of ten of these mutants with penicillin were made. Six of the curves were no different from those of the parent strain *B/p* but four gave curves identical with the penicillin survival curves of strain *B/r/p*. In these four strains, the resistance to penicillin was shifted to a higher level by a mutation to radiation-resistance, acting as a modifier of an independent mutation to penicillin-resistance.

Some Preliminary Experiments on Population Dynamics

The study of the factors governing selection in mixed populations of bacteria is limited, at the present time, by our inadequate knowledge of the genetic makeup of bacteria, and by the complexity of their physiological responses under conditions which are not strictly defined. To the extent that well-defined and easily detected mutants are available within a strain, however, it may become profitable to explore the behavior of artificially mixed populations of known constitution.

The method used in the preliminary experiments to be described below consisted of (1) the preparation of mixtures of various proportions of strains *B* and *B/r*, (2) the maintenance of the mixed cultures under specified conditions, and (3) the periodic determination of the proportions of each strain present in the mixtures.

In the first series of experiments, four duplicate sets of mixed cultures were set up by inoculating measured volumes from fully grown broth cultures of strains *B* and *B/r* simultaneously into tubes of fresh broth. Immediately after the inoculation of the mixed cultures, assays were made to determine precisely their composition. Each culture was assayed in two ways: first, to determine the total number of bacteria, both *B* and *B/r*, per ml of the culture; second, to determine the number of resistant bacteria per ml of the mixture. The assays for the total number of bacteria were conducted by the usual method of dilution, plating and colony counts. The assays for the number of resistant bacteria were made by subjecting samples from the mixed cultures to the double-irradiation treatment. Knowing the titre of the culture as a whole, and the titre of the *B/r* component, the proportion of mutant bacteria in each of the cultures was computed.

In the first experiment, the mixtures were incubated at 37°C, and were serially subcultured in broth every 24 hours over a period of 24 days. Every

48 hours, the proportion of resistant bacteria in the current set of subcultures was determined, using the two assay methods described above. Figure 4 shows the proportion of resistant bacteria in each of the mixtures plotted as a function of time in days after the start. The curves show that no significant change in the proportions of the two strains occurred over the 24-day period. This experiment was repeated, using synthetic medium throughout instead of nutrient broth, and the results were essentially the same.

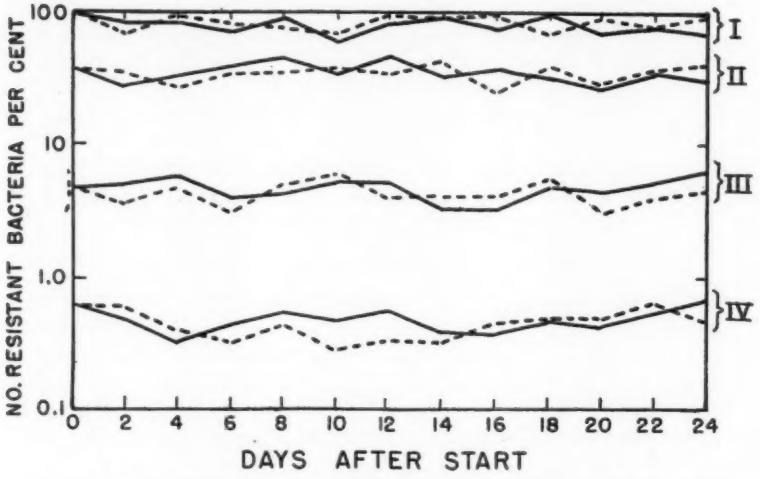

FIGURE 4.—Proportion of resistant bacteria in artificially mixed cultures of strains *B* and *B/r*, maintained by daily serial subculture.
(I) duplicate mixtures—initial proportions 1.0% *B*:99.0% *B/r*
(II) duplicate mixtures—initial proportions 37.0% *B*:63.0% *B/r*
(III) duplicate mixtures—initial proportions 95.1% *B*: 4.9% *B/r*
(IV) duplicate mixtures—initial proportions 99.4% *B*: 0.6% *B/r*

A second experiment was conducted, in which the method of maintaining the mixed cultures was modified. In this experiment, only one set of mixtures was followed, again in duplicate. The initial proportions of this set of mixtures, which were carried in nutrient broth, were approximately 80% *B/r*: 20% *B*. As in the first experiment, the mixtures were subcultural serially every 24 hours, and periodic determinations were made of the proportion of mutant bacteria. However, the initial mixed cultures, from which the series of subcultures was started, were not discarded. Instead, they were placed in the incubator, and allowed to age at 37°C for the 20-day period of the experiment. Determinations of the proportion of resistant bacteria in these aging cultures were made every 48 hours. The results of this experiment are shown in figure 5. When the mixtures were carried by daily serial subculture, as in the first experiment, no significant change in the proportion of resistant bacteria was

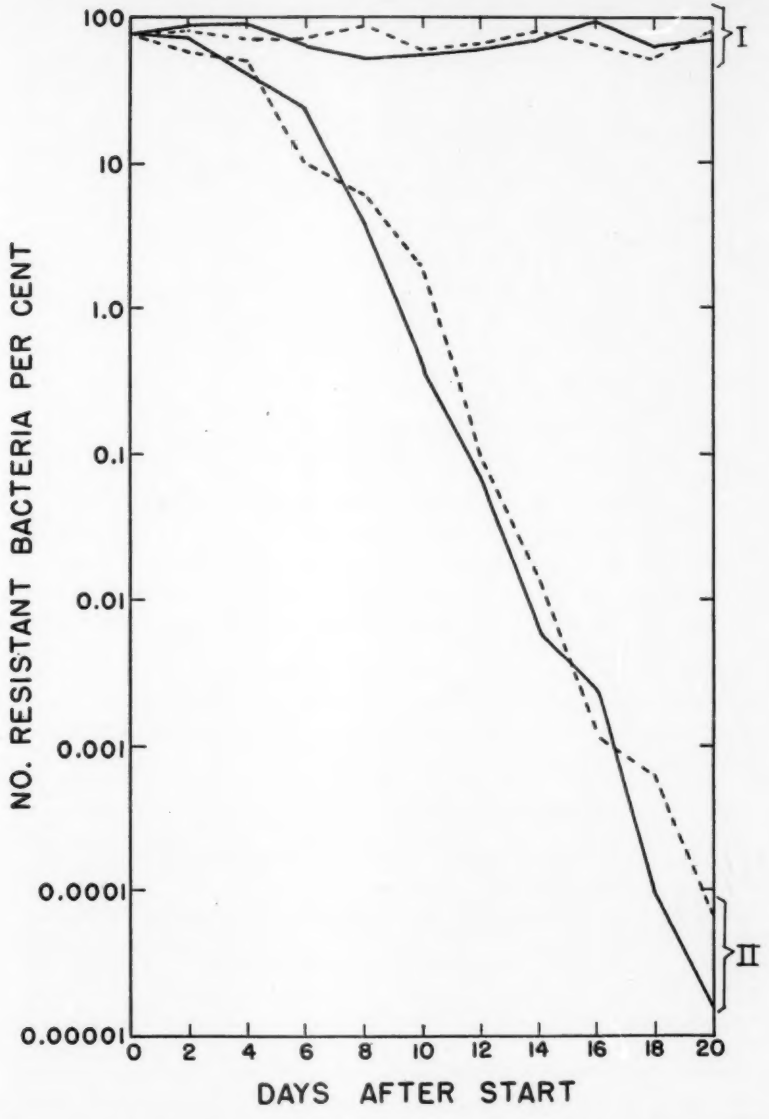

FIGURE 5.—Proportion of resistant bacteria in artificially mixed cultures of strains *B* and *B/r*.
(I) duplicate mixtures: initial proportions 20% *B*; 80% *B/r* maintained by daily serial subculture.
(II) same duplicate mixtures as (I), maintained by aging without subculture.

observed. When the cultures were allowed to age without subculture, the proportion of the *B/r* component dropped from 80 percent to about 0.00005 percent, or 1 in 2×10^6 bacteria, in 20 days. There are indications that the proportion of mutants continues to fall, until there are no mutants in a sample of 10^8 bacteria.

These results indicate that, under the conditions of aging broth cultures, strain *B/r* is at a distinct selective disadvantage in mixture with strain *B*. No such disadvantage is manifest under the conditions of daily serial transfer to fresh medium.

DISCUSSION

Evidence has been presented that the increased resistance to radiation exhibited by strain *B/r* is the result of a spontaneous mutation occurring in cultures of the parent strain. At least four separate mutations have been differentiated, each of which results in resistance to both ultraviolet and x-rays. but distinguishable on the basis of secondary resistance to penicillin and sulfathiazole. The estimated mutation rate, 10^{-5} mutations per bacterium per generation, should therefore be interpreted as the sum of at least four separate mutation rates.

The mutation rate was calculated on the simplifying assumption that the growth rates of mutant and parent bacteria are the same, under the experimental conditions. The dangers inherent in this assumption are evident, particularly in the light of the population dynamics experiments. These experiments show that, under different conditions of mixed culture, the relative growth rates of mutant and parent bacteria may vary widely. Estimates of mutation rates which are based on assumptions as to growth rates under given conditions may, therefore, involve considerable error.

Although mutations to radiation-resistance occur spontaneously, their rate can be increased considerably by treatment with high doses of ultraviolet. The fact that the inducing agent and the specific selective agent for the mutant are the same constitutes an obstacle to the quantitative study of the induction effect. This difficulty, however, has an interesting philosophic implication. The production of radiation-resistant mutants by radiation, along with other seemingly purposive biological events, might be interpreted by some as a manifestation of teleology. The spurious nature of such an explanation is easily exposed in this case, since it is known (1) that radiation-resistance occurs spontaneously, as well as in direct response to radiation, and (2) that radiation-resistance is only one of a number of random changes induced by radiation, although it is automatically selected by the treatment responsible for its induction.

Speculations as to the possible mechanism of resistance to radiation must be based upon a more or less definite conception of the manner in which radiation kills bacteria. The target theory (for general discussions see TIMOFÉEFF-RESSOVSKY 1937, ZIMMER 1943, LEA 1946) has provided a basis for one interpretation of the bactericidal action of radiation. This theory, which has been applied extensively to radiobiological phenomena and is supported by con-

siderable experimental evidence, will be adopted tentatively as a starting point in this discussion.

According to the simplest assumptions of the target theory, certain biological effects of radiation (in this case, the killing of bacteria) are produced whenever a "hit" or a specific number of "hits" occurs in a sensitive region, or target. A hit is defined as an elementary act of absorption of radiation energy, that is, as the production of an ionization (in the case of x-rays) or as the absorption of a photon (in the case of ultraviolet).

In the application of the target theory to the killing of bacteria by radiation, the interpretation of survival curves has played an important part. When the fraction of surviving bacteria is plotted against the dose, it is often found that each successive increment of dose kills the same *proportion* of the remaining bacteria, rather than the same *number* of bacteria. In other words, when the logarithm of the surviving fraction is plotted as a function of the dose, a straight line is obtained. Exponential survival curves of this type have been described for ultraviolet by WYCKOFF (1932), HERČÍK (1936), HOLLAENDER and CLAUS (1936), and LEA and HAINES (1940).

The central features of the target theory rest on the interpretation of the statistical law underlying the exponential curves:

$$\log N/N_0 = kD,$$

where N_0 is the initial number of bacteria, N is the number of bacteria surviving and D is the dose. The dose D, according to the target theory, represents ultimately the number of hits occurring per unit of volume, and therefore defines the probability that a hit will occur in a sensitive volume, or target. The constant k thus directly represents the size of the target, and a single hit, occurring within the target, will kill a bacterium. Certain investigators (COBLENTZ and FULTON 1924, GATES 1929, DREYER and CAMPBELL-RENTON 1936) have reported survival curves which are sigmoid in shape. Sigmoid survival curves are regarded with suspicion by some proponents of the target theory (LEA 1946), on the ground that they are obtained only when certain sources of experimental error are not rigorously controlled. *Bona fide* sigmoid curves, however, would be interpreted as signifying that two or more hits in a sensitive volume are required to kill a bacterium.

Within the framework of the target theory, how can a mutation leading to increased resistance to radiation be explained?

The survival curves for strain B, with both ultraviolet and x-rays, are exponential. Let us assume, then, that sensitive bacteria are killed whenever a single hit occurs in a sensitive volume. The ultraviolet survival curve for the resistant strain is distinctly sigmoid in shape. Technical sources of error cannot be responsible for this deviation from exponential killing, since the techniques used were identical with those involved in the preparation of the corresponding curve for strain B. This curve must be interpreted, according to the target theory, as a multiple-hit curve, indicating that several hits in a sensitive volume are required to kill a resistant bacterium. The ultraviolet curves suggest that the mutation from sensitivity to resistance changes the

mode of killing from a one-hit to a multiple-hit mechanism.

The x-ray curve for strain *B/r*, however, is a one-hit curve, differing only in slope from the x-ray curve for the sensitive strain. Since a single x-ray hit supplies considerably more energy than a single ultraviolet hit (the ratio being approximately 6:1), an effect requiring multiple ultraviolet hits can be satisfied by a single x-ray hit (LATARJET 1946). However, if the mutation involves merely a change in the energy threshold for killing which is still satisfied by a single x-ray hit, the mutant should not exhibit increased resistance to x-rays. On the basis of the x-ray data, the mutation seems to produce a decrease in the size of the target (which determines the value of *k*, and hence the slope of the curve). Or, if it is assumed that a number of possible targets exist in sensitive bacteria, a single hit in any one of which will result in death, the change may involve the inactivation of a fraction of the available targets.

In any case, there is a contradiction between the x-ray and ultraviolet data, which are not easily reconcilable according to the simplest assumptions of the target theory.

The validity of the assumption that the value of *k* is determined by the size of the sensitive volume alone, and that a hit within the target is always effective, has recently been questioned on theoretical grounds by a number of investigators (SOMMERMEYER 1938, JORDAN 1939, MULLER 1940, FANO 1942). These authors have discussed, as an additional factor to be considered as influencing the value of *k*, the probability that a hit occurring in the sensitive volume will produce a given effect. This "quantum efficiency" factor which has been applied in experimental work by WOLLMAN, HOLWECK and LURIA (1940), and by LATARJET (1946), can approach one, or can be very small, depending upon the reaction system involved. Taking into account this probability factor in determining the value of *k*, a consistent explanation of resistance to ultraviolet and x-rays can be developed.

Let us assume that, in the sensitive strain, a single ultraviolet hit will, with a certain probability, produce a primary change which will lead to the death of the bacterium. A single x-ray hit is also effective, with its own probability. Suppose, then, that the mutation to resistance raises the threshold for the production of the primary effect, so that the probability that a single ultraviolet hit will be effective approaches zero. A multiple-hit mechanism for the killing of resistant bacteria with ultraviolet may result. A single x-ray hit may still supply the energy required to meet the new threshold, so that a single-hit curve will be obtained with x-rays, *but the change in threshold decreases the probability that an x-ray hit will be effective, so that the slope of the curve, determined by k, is shifted in the direction of greater resistance.*

The parallel radiobiological results obtained for the production of mutations in Drosophila and the killing of bacteria, among other considerations, have led to the suggestion that the bactericidal effect of radiation may be due to the induction of lethal mutations (LEA and HAINES 1940, LEA 1946). On the basis of this hypothesis, the change in threshold for the production of the primary effect of radiation, discussed above as a possible result of the mutation from sensitivity to resistance, can be given a more concrete formulation.

The mutation can be envisaged as a rearrangement of the gene, or of a critical constituent of it, leading to greater stability, and consequently to decreased susceptibility to induced lethal mutation. In Drosophila, many mutated genes are known to be less susceptible to the inducing action of radiation than the original alleles (TIMOFÉEFF-RESSOVSKY 1933).

Certain effects of radiation which lie outside the scope of the target theory can be considered as a basis for an entirely different approach to the mechanism of resistance. DALE (1940, 1942) has developed the argument that the inactivation of enzymes may be important in the lethal action of radiation. Another possibility, mentioned by LEA (1946), is that the decomposition products of proteins, or other cell constituents, produced as a result of irradiation, may act as cell poisons. With these suggestions in mind, the following observations take on significance for the problem under discussion: (1) All radiation-resistant strains are resistant to the division-inhibiting action of radiation as well as to its lethal effect, (2) many radiation-resistant strains are also resistant to penicillin, or sulfathiazole or both; (3) strains resistant to sulfathiazole and penicillin are resistant to the division-inhibiting action of these agents as well as to their lethal effects.

These facts suggest a direct relationship between the division-inhibiting action of these antibacterial agents and their lethal effect. LEA, HAINES and COULSON (1937) have presented evidence that these two effects of radiation are independent of one another. The observation that resistance to the division-inhibiting effect of ultraviolet invariably accompanies resistance to its bactericidal action is evidence that the two effects are closely related, unless the unlikely assumption is made that the mutation from B to B/r involves two independent changes.

The microscopic study of sensitive bacteria irradiated with low doses of ultraviolet yields two further significant observations: (1) bacteria which recover from the division-inhibiting effect of the treatment within a certain time give rise to colonies, that is, survive; (2) bacteria which do not recover from the division-inhibiting action of the treatment within a certain time do not give rise to colonies, that is, are killed. Thus, recovery from the inhibition of division produced by radiation seems to be associated with survival. The resistance of strain B/r to radiation may be due to a change which renders this strain less susceptible to inhibition of division, or more efficient in recovery. The shorter lag phase characteristic of radiation-resistant strains may be another manifestation of this property.

The inhibition of division which occurs as a result of treatment with radiation may be due to either (a) the accumulation of an inhibitor (cell poison hypothesis), or (b) the destruction of a compound necessary to division (enzyme inactivation hypothesis). The resistance of strain B/r may be due to its greater efficiency in either the reformation of the inactivated compound or in the destruction or neutralization of the inhibitor.

The resistance of certain radiation-resistant strains to penicillin and sulfathiazole can be considered in this connection. Although the primary actions of these substances are likely to differ from each other, and from the primary

action of radiation, all of these agents share the ability to inhibit division in sensitive cells, and to a lesser extent in resistant bacteria. The chains of reactions blocked by the primary action of each of these agents may share one or more common steps. Different mutations to resistance may alter, or provide alternative pathways for, different steps in the reaction chains. When a step common to all of the reaction chains is affected, resistance to all of these agents may result.

One experimental observation, which was briefly mentioned in an earlier section, may have some bearing on the present discussion. Figure 2 shows two sets of ultraviolet survival curves for strains *B* and *B/r*, one set obtained when the bacteria were irradiated on the surface of solid medium, and the other when the irradiation was made in a nonabsorbing synthetic liquid medium. Both strains show greater sensitivity when irradiated on solid medium. Microscopic examination showed that bacteria irradiated on solid media exhibit more prolonged inhibition of division than bacteria treated in liquid suspension. This correlation between sensitivity and susceptibility to inhibition of division is further evidence of the intimate relation between these two factors.

Differences in treatment of the bacteria before and after irradiation, as well as differences in specific components of the media, have been eliminated as the factors responsible for this effect. Reflected radiation from the agar surface has also been eliminated (HOLLAENDER 1946). The difference in sensitivity seems to depend solely upon the liquid or solid character of the medium. This suggests the possibility that cell poisons, produced in the bacteria as a result of irradiation, diffuse away more rapidly in the liquid than in the solid medium, resulting in higher survival under the former conditions, and earlier recovery from the division-inhibiting effect.

As advances are made in our understanding of the pathways through which which radiation exerts its lethal effect, more fruitful approaches to the mechanism of resistance to radiation may become available.

Finally, some remarks will be made concerning the preliminary experiments on population dynamics described above. It will be remembered that, under the conditions of daily subculture, the proportion of mutants in mixture with sensitive bacteria was maintained at the initial level over a period of 24 days. When the mixtures were allowed to age in the incubator, however, the proportion of mutants dropped sharply, approaching complete elimination during the period of the experiment.

In mixed cultures maintained in broth at 37°C, selection apparently does not operate against either strain during the early phases of growth. The stage of growth during which selection is effective against the mutant strain is the so-called "stationary" phase. During this phase, according to JORDAN and JACOBS (1944), the viable count remains constant, while the total count increases steadily. The stable viable count is thus determined by a balance between the rates of division and death. BRAUN (1946) found that selection during this phase of growth was responsible for the characteristic dissociation patterns in clones of *Brucella abortus*.

The theoretical influence of selection pressure, and of forward and reverse

mutation rates, in determining the equilibria reached in genetically mixed populations has been analyzed mathematically by WRIGHT (1931), and for bacteria specifically by DELBRÜCK (1945). In general, equilibria in cultures of strains having stable properties are determined by forward mutation rates, which may be opposed by reverse mutation, or by selection against the mutant, or both. DELBRÜCK has pointed out that mutations occurring at very low rates need not be opposed by either of these factors, since the probability is low that rare mutants will be carried over at each subculture of the stock. Mutations occurring at high rates, however, must be opposed by selection or reverse mutation or both, if the original stock is to maintain its constancy.

Under the conditions of aging broth cultures, there is no evidence that forward mutation opposes the selection against radiation-resistant mutants, with the establishment of an equilibrium. If mutation is dependent upon the occurrence of cell division, the apparent absence of mutation pressure may be related to the rarity of dividing bacteria in aging cultures.

These experiments suggest that, in the case of mutations occurring at high rates, the method of carrying the stock can be important in maintaining the constancy of its properties. If selection is operative against such a mutant only in the stationary phase of growth, the mutant would soon replace the parental type (in the absence of reverse mutation) if the stock should be carried by daily serial subculture. The mutant could be held in check, however, if the stock culture were permitted to age between subcultures.

The rate of mutation to radiation-resistance, which is of the order of 10^{-5} mutations per bacterium per generation, is apparently not sufficiently high to permit the establishment of the mutant, even under conditions of daily serial subculture of the parent stock.

Further experiments are being conducted to determine the relation between the observed selection rate and the rates of division and death of the parent and mutant strains, in pure culture and in mixture. The dependence of the selection rate upon various initial proportions of parent and mutant bacteria, and upon specific conditions of growth and constituents of the medium, seems also worthy of investigation, since the experimental control of selection pressure in mixed populations of bacteria is likely to be of primary practical importance. The value of bacteria as materials for population studies, for which they are uniquely suited in many ways, will increase with our knowledge of their genetic and physiological constitution.

SUMMARY

Strain *B* of *Escherichia coli* gives rise to a variant which is characterized by relative resistance to the lethal effects of both ultraviolet radiation and x-rays. This character is stable and heritable.

Some additional properties of radiation-resistant strain, *B/r*, derived from the parent strain by culturing a last survivor of an irradiation with ultraviolet, are described. This strain exhibits relative resistance to the division-inhibiting effect of radiation, as well as to its lethal effect, and is also relatively resistant to the lethal and division-inhibiting action of penicillin and sodium sulfathia-

zole. Strain *B/r* exhibits a shorter lag phase in broth at 37°C than the parent strain, and a somewhat lower final titre. Survival curves of strains *B* and *B/r* with ultraviolet radiation and x-rays are compared.

A technique is described whereby radiation resistant mutants can be detected quantitatively in samples from the parent strain. This technique, designated as the double-irradiation treatment, provides an absolute selective procedure for the detection of the variant.

Resistance to radiation is shown to be due to a spontaneous mutation, occurring in cultures of strain *B* at a rate of about 10^{-5} mutations per bacterium per generation.

The rate of occurrence of the mutation to radiation-resistance can be increased considerably by treatment of the parent strain with high doses of ultraviolet radiation.

At least four independent mutations are demonstrated, each of which results in resistance to radiation, but distinguishable by the presence or absence of associated resistance to penicillin, or sodium sulfathiazole or both.

The mutation to radiation-resistance, when it is accompanied by resistance to penicillin, acts as a modifier of an independent mutation to penicillin-resistance, adding quantitatively to its effect.

Some preliminary experiments in population dynamics are described. When strains *B* and *B/r* are artificially mixed and carried by daily subculture, neither strain is at a selective disadvantage. When the mixtures are aged without subculture, however, the mutant strain is at a distinct selective disadvantage approaching complete lethality.

Interpretations are offered for certain features of the results.

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MUTATIONS TO SULFONAMIDE RESISTANCE IN *STAPHYLOCOCCUS AUREUS*[1]

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TWO general hypotheses have been postulated to explain the origin of drug-resistant strains of bacteria. These hypotheses are: (1) Resistant cells arise by mutation and are selected when grown in the presence of drugs. (2) Resistance is the result of a specific adaptation of bacteria in response to inhibiting agents.

Resistance of *Escherichia coli* to bacteriophage (LURIA and DELBRÜCK, 1943) resistance of *Staphylococcus aureus* to penicillin (DEMEREC 1945), and resistance of *Escherichia coli* to ultraviolet radiation (WITKIN 1946) have been shown to be the result of mutations in individual cells. These mutant cells and clones formed from them possessed selective advantages in the presence of the destructive agent concerned. It is generally accepted that naturally occurring strains of sulfonamide-sensitive species of bacteria exhibit low tolerance for these drugs (SCHMIDT *et al.*, 1940; FRISCH 1942; SCHMIDT *et al.* 1943; LANDY and GERSTUNG 1945; BERNHEIMER 1946). Resistance to sulfonamides is characteristic of a large number of bacterial cultures isolated from patients who have undergone sulfonamide therapy (FRISCH 1942; SCHMIDT *et al.* 1942; EPIDEMIOLOGY UNIT NO. 22, 1945; LANDY and GERSTUNG 1945; BERNHEIMER, 1946). However, the mechanism by which sulfonamide fastness is acquired is not clearly understood. STRAUSS *et al.* (1941a and b), SESLER and SCHMIDT (1942), KIRBY and RANTZ (1943), and SCHMIDT and SESLER (1943) reported that sulfonamide tolerance is the result of physiological adaptation of bacteria, but their experiments do not give conclusive evidence for this mechanism. LANKFORD *et al.* (1943) and SPICER (1945) concluded that naturally occurring bacterial populations are a mixture of cell types; sulfa drugs act merely as selective agents in the development of resistant strains. No data were given on the origin of these few resistant cells or on their rate of occurrence.

As the mode of origin of sulfonamide-resistant strains of bacteria is subject to debate, it was felt that this phenomenon needed additional clarification. Such information would be valuable in therapy, for if mutation and selection would prove to be the mechanisms involved, doses of sulfonamides should be high enough to inhibit either the few resistant cells that may be present in a sensitive bacterial strain or resistant cells that may arise by mutation. Also,

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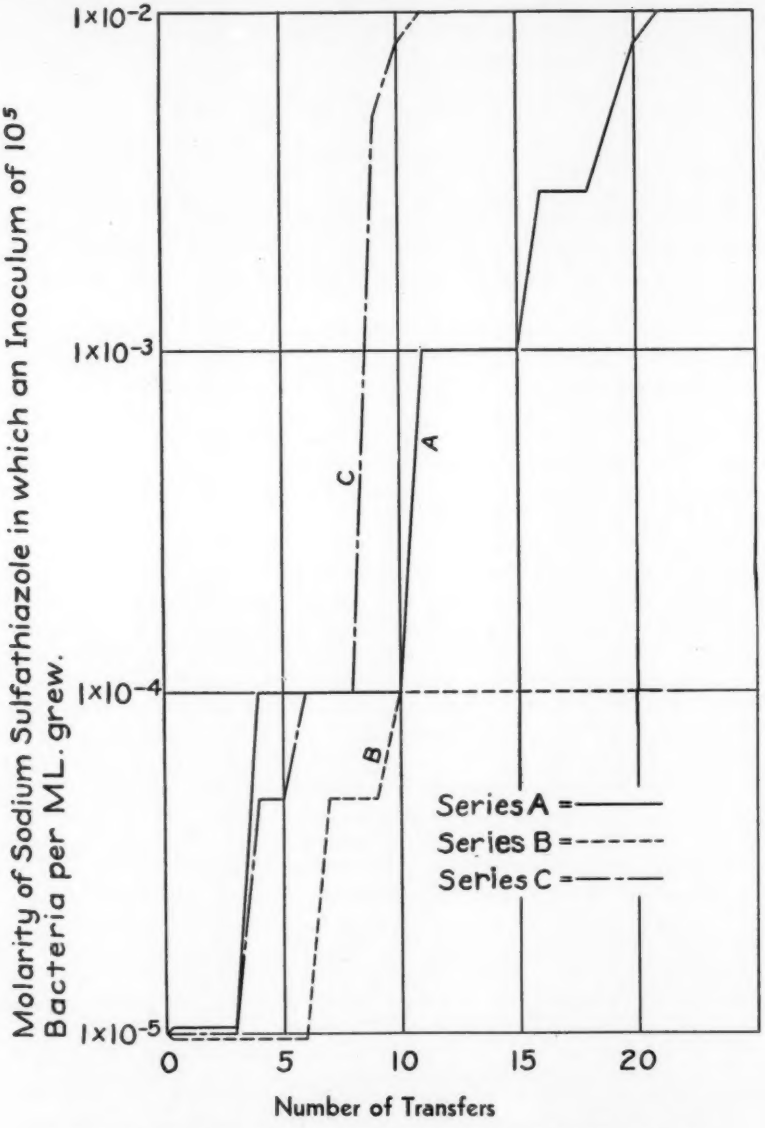

FIGURE 1.—Resistance of *S. aureus* during serial transfer in Sodium Sulfathiazole.

as the action of sulfonamides has been associated with specific cell processes—especially *p*-aminobenzoic acid metabolism—it was hoped that changes in physiological characteristics could be correlated with the change from sensitivity to resistance.

MATERIALS

The strain of *Staphylococcus aureus* used was obtained originally from the NORTHERN REGIONAL RESEARCH LABORATORY, Peoria, Illinois. This same strain, designated as NRRL-313 was used by DEMEREC (1945) in a study of mutations to penicillin resistance.

Sodium sulfathiazole (NaST) was chosen because of its high antibacterial activity and because of its high solubility. As strains resistant to one sulfonamide usually show cross-resistance with most sulfa derivatives, with the exception of sulfamylon (SCHMITH 1943), the experiments were confined to one drug. NaST was prepared in 1×10^{-1} M solution in distilled water and diluted to the desired concentrations.

A semisynthetic casein hydrolysate medium described by STRAUSS *et al.* (1941b) was used in order to avoid sulfonamide antagonists present in ordinary bacteriological media. Twenty-five gms of powdered agar were added per 1900 ml of medium for pouring plates.

DEVELOPMENT OF RESISTANT STRAINS BY SERIAL TRANSFER

Initial attempts to establish resistant strains by isolation of the colonies that developed when a sensitive culture was plated in sulfonamide-agar were precluded by the effects of inoculum size. Large inocula gave complete growth even in agar saturated with NaST. No colonies developed when the number of organisms plated was small enough for inhibition of bacterial growth. Therefore, preliminary experiments were based upon observation of the magnitude and distribution of increases in resistance that occurred during serial transfer in liquid medium containing NaST.

1×10^{-5} M was the maximum concentration of NaST in which an inoculum of 10^2 or 10^3 cells per ml of strain NRRL-313 grew. At each transfer inocula of 10^6, 10^5, 10^4, 10^3, and 10^2 bacteria per ml of culture were tested with a range of 1×10^{-2} to 1×10^{-5} M NaST. Growth was estimated from turbidity after 48 and 72 hours incubation at 37°C. Bacteria for the next transfer were taken from the highest concentration of NaST in which an inoculum of 10^5 cells per ml had grown to almost full turbidity in 72 hours. This inoculum was selected because it was inhibited by the same NaST molarities as inocula of 10^3 and 10^2 cells per ml, yet it was large enough to allow detection of resistant cells. As resistance of the strains increased the higher concentrations of NaST were spaced at closer intervals, and some of the lower concentrations were omitted.

Results of three transfer series, A, B, and C are given in figure 1. A total increase in NaST tolerance of 1,000 times occurred in series A and C, and an increase of ten times occurred in series B. The characteristic resistance of each

resultant strain was maintained through 15 transfers in the absence of NaST, and therefore appeared to be a stable, heritable property.

Increases in resistance appeared as discrete steps that were of different magnitude and were separated by flat plateaus. The number of transfers separating the steps differed both between different series and between successive increases within the same series. It is possible that the steps observed were somewhat forced by the NaST concentrations used. The data do not support this hypothesis, for in nine instances intermediate NaST concentrations were bypassed at a single transfer.

Stepwise increases in resistance occurring at random time during the course of serial transfers, and stability of resistance in the absence of sulfonamides, are better explained by mutation and selection than by physiological adaptation. The resistant mutants that arise in a sensitive strain possess selective advantages when grown in the presence of sulfonamides, and should constitute a large proportion of the inoculum used for the next transfer. Consequently, serial transfer in liquid medium is unsuitable either for estimation of mutation rates or for isolation and study of individual mutants.

DERIVATION OF RESISTANT STRAINS BY COLONY ISOLATIONS

When Seitz filtrates from cultures in NaST were tested, it was observed that a reduction of sulfonamide activity had occurred even when visible growth was not reached. No loss of NaST could be demonstrated by colorimetric titration, but this method may not have given a selective differentiation between biologically active and biologically inactive sulfonamide molecules. Another hypothesis is suggested by the observation of ROSE and FOX (1942) that bacteria exposed to sulfonamides undergo a certain number of divisions before growth is stopped. Production of antagonists during this noninhibited phase of bacterial growth would result in reduction of effective sulfonamide concentrations. The relation of antagonist production to growth also was indicated by the absence of inoculum-size effects when the bacteria tested previously had been exposed to inhibitory concentrations of NaST. CHANDLER and JANEWAY (1939) and LOWELL *et al.* (1941) made similar observations. Utilization of inhibited bacteria therefore should allow the plating of inocula large enough for the detection of rare resistant cells.

The following technique was used for the isolation of resistant bacteria from strain NRRL-313. Each of ten tubes containing ten ml of casein hydrolysate broth was inoculated with approximately 10^2 sensitive bacteria from a 24-hour culture. These tubes were incubated for 48 hours at 37°C. From each of these ten cultures grown from small inocula a 3.5-ml culture containing 1×10^{-4} M NaST was inoculated with 10^6 cells per ml. These NaST cultures had titers between 1 and 2×10^8 cells per ml after 18 hours of incubation at 37°C instead of the 8 to 10×10^8 cells per ml observed in control cultures. These inhibited bacteria were tested in plates containing a final concentration of 3×10^{-5} M NaST. One ml of each undiluted culture was poured into each of three test plates. Three 1-ml portions of a 10^{-6} dilution were plated in agar without NaST for assay of the bacterial titer.

All colonies that developed in NaST agar were counted, and samples from each culture were transferred to 5-ml tubes containing 1×10^{-5} M NaST. Bacteria from strain NRRL-313 grew slowly in this concentration of NaST, while resistant mutants were not inhibited. Thus overgrowth of the resistant cells by more sensitive, faster-growing organisms was prevented. As 1×10^{-5} M represented the minimum inhibitory concentrations of NaST, selection pressure in favor of secondary mutants with still higher resistance should have

TABLE 1

Resistance of mutants from different strains of S. aureus.

MAXIMUM MOLAR CONCENTRATION OF NaST IN WHICH MUTANT GREW*	PARENT STRAIN	SUBSTRAINS FROM NRRL-313		SUBSTRAIN FROM STRAIN 38
	NRRL-313, RESISTANT TO 1×10^{-5} M NaST	STRAIN 33, RESISTANT TO 5×10^{-5} M NaST	STRAIN 38, RESISTANT TO 1×10^{-4} M NaST	STRAIN 67, RESISTANT TO 1×10^{-3} M NaST
$5\text{–}6^{**}\times10^{-5}$	1			
$1\text{–}2\times10^{-4}$	15	19		
$3\text{–}4\times10^{-4}$	1	0	12	
$5\text{–}6\times10^{-4}$		4	4	
$9\text{–}10\times10^{-4}$		7	18	
2×10^{-3}				6
3×10^{-3}				6
4×10^{-3}				5
Total number of independent mutants isolated and tested	17	30	34	17

* Test inoculum 10^3 cells per ml of final culture.

** Variation between different experiments necessitates expression of NaST tolerance as a range for most strains.

been minimized. The resulting cultures were tested by adding approximately 10^3 bacteria per ml to 2-ml broth tubes containing varied NaST concentrations. Resistant strains were established on nutrient agar slants from tubes where complete growth had occurred in concentrations of NaST higher than 1×10^{-5} M.

Similar techniques were used for isolation of highly resistant mutants from strains obtained from NRRL-313. NaST concentrations were increased five times for strain 33, ten times for strain 38, and 100 times for strain 67.

NaST resistance of the mutant strains

Resistance of the substrains isolated is given in table 1. Variation of individual cultures in different tests made it necessary to evaluate resistance in terms of a range of NaST concentrations; the values given represent maximum

tolerance as measured by at least three different tests. Strain NRRL-313, resistant to 1×10^{-5} M NaST, gave mutants resistant to $5\text{–}6\times10^{-5}$, $1\text{–}2\times10^{-4}$, and $3\text{–}4\times10^{-4}$ M NaST. Strain 33, a substrain from NRRL-313 that was resistant to 5×10^{-5} M NaST, gave mutants resistant to $1\text{–}2\times10^{-4}$, $5\text{–}6\times10^{-4}$, and $9\text{–}10\times10^{-4}$ M NaST. Strain 38, a substrain from NRRL-313 that was resistant to 1×10^{-4} M NaST, gave mutants resistant to $3\text{–}4\times10^{-4}$, $5\text{–}6\times10^{-4}$, and $9\text{–}10\times10^{-4}$ M NaST. Strain 67, a substrain from strain 38 that was resistant to 1×10^{-3} M NaST, gave mutants resistant to 2×10^{-3}, 3×10^{-3}, and 4×10^{-3} M NaST. Increases from strain 67 seem small, but these bacteria already had undergone two mutations, each of which had resulted in an increase of ten times in NaST tolerance. Also, the actual amount of sulfonamide

TABLE 2

Test for sulfonamide-resistant colonies in ten "inhibited" cultures of NRRL-313.

CULTURE	TITER OF CULTURES* ($\times10^8$)	COLONIES IN TEST PLATES			TOTAL COLONIES
		PLATE 1	PLATE 2	PLATE 3	
A	1.6	0	0	0	0
B	1.1	13	11	11	35
C	1.5	32	40	39	111
D	1.5	0	0	0	0
E	0.9	0	0	0	0
F	2.3	0	0	0	0
G	2.4	0	0	0	0
H	1.5	2	8	10	20
I	2.8	6	4	3	13
J	2.1	2	2	1	5

* Mean of three assay plates.

involved in changing from 1×10^{-3} to 4×10^{-3} M concentrations is much greater than for any previous step. These results indicate that the few colonies that grow when a large inoculum is plated in NaST agar have increased resistance to NaST. Variants with even higher resistance may be obtained from the strains first isolated.

Origin of resistant bacteria

Whether the resistant variants arise by mutation or by physiological adaptation may be tested by the method proposed by LURIA and DELBRÜCK (1943). If cultures are started from an inoculum small enough to make the inclusion of resistant cells highly improbable, random variation in the occurrence of rare mutations should result in statistically significant fluctuations in the number of resistant cells in different cultures. No significant differences between cultures would be expected if the process involved is one of adaptation.

Data for a typical experiment are given in table 2. The fact that some

cultures contained no resistant cells while other cultures contained many is conclusive evidence that resistance was not the result of physiological adaptation.

The postulate that mutations arise at random time during the growth of cultures was tested by variance analysis of those cultures that gave colonies in each of three test plates (table 3). The omission of cultures with no colonies

TABLE 3

*Variance analysis of the number of colonies per culture.**

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE
Total	14	
Between cultures	4	614.05**
Between plates within cultures	10	8.07

* Data from Table 2, limited to cultures that showed colonies.
** Significant at one percent level.

TABLE 4

Significance of variation in number of resistant cells in different cultures.

CULTURES USED AS PARENT STRAINS	NUMBER OF EXPERIMENTS AVAILABLE FOR STATISTICAL TEST*	SIGNIFICANCE OF BETWEEN-CULTURE MEAN SQUARES		
		NO SIGNIFICANT DIFFERENCES BETWEEN CULTURES	DIFFERENCES BETWEEN CULTURES SIGNIFICANT AT 5% LEVEL	DIFFERENCES BETWEEN CULTURES SIGNIFICANT AT 1% LEVEL
NRRL-313	2	0	0	2
Strain 33	4	0	0	4
Strain 38	2	0	1	1
Strain 67	1	0	0	1
Totals	9	0	1	8

* Analysis limited to experiments with three or more cultures that gave resistant colonies on each of three plates per culture.

minimizes differences between cultures. Nevertheless, variation in the number of resistant cells per culture was highly significant. The results for nine independent experiments are given in table 4. Variation between cultures was significant at the one percent level in eight experiments and significant at the five percent level in one experiment. This highly significant fluctuation in the number of resistant cells in different cultures started from a common source is strong evidence that resistant bacteria arise by mutation.

Mutation rates

The rate of mutation from sensitivity to resistance was estimated from the percentage of cultures not giving resistant colonies (LURIA and DELBRÜCK 1943). Data for strain NRRL-313 and three substrains from NRRL-313 are given in table 5. Different classes of resistant mutants were pooled to form the total for each individual strain. The four strains tested all gave similar mutation rates, with a range of 2×10^{-9} to 4×10^{-10} per bacterium per generation. This close agreement between strains, even though some had undergone two previous mutations, suggests a large number of different mutations to NaST resistance.

The minimum number of mutations concerned probably is five; the maximum number may be considerably larger. The minimum figure is based on

TABLE 5

Mutation rates of different strains of S. aureus.

PARENT STRAIN	NaST RESISTANCE OF PARENT STRAINS	TOTAL NUMBER OF CULTURES	NUMBER OF CULTURES WITHOUT MUTANTS	FRACTION OF CULTURES WITHOUT MUTANTS	MEAN NUMBER OF BACTERIA TESTED PER CULTURE ($\times10^8$)	MUTATION RATE PER BACTERIUM PER GENERATION
NRRL-313	1×10^{-5}	50	34	0.68	6.9	4×10^{-10}
Strain 33	5×10^{-5}	40	12	0.30	4.3	2×10^{-9}
Strain 38	1×10^{-4}	40	19	0.48	6.4	8×10^{-10}
Strain 67	1×10^{-3}	40	21	0.53	5.0	9×10^{-10}

strain 67, which gave three classes of mutants after having undergone two previous mutations to NaST resistance. Except for increased *p*-aminobenzoic acid production, no criterion was found for differentiation of mutants from different strains. Equal magnitude of resistance changes cannot be used, for it is impossible to determine how the same mutation may affect the phenotype when it occurs in cells of different genetic constitution.

PRODUCTION OF *p*-AMINOBENZOIC ACID BY RESISTANT STRAINS

According to the WOODS-FILDES hypothesis (WOODS 1940) that sulfonamides act through competition for an essential metabolite—specifically, *p*-aminobenzoic acid (PAB)—increased PAB synthesis could account for increased bacterial resistance. LANDY, et al. (1943) reported that sufficient PAB was present in the filtrate of a sulfonamide-resistant culture of *Staphylococcus aureus* to explain the level of resistance observed. However, LANDY and GERSTUNG (1945) found that while a high percentage of sulfonamide-fast strains of gonococci showed increased PAB synthesis, some resistant strains produced very little PAB. Conversely, some sensitive strains produced more PAB than others. Therefore, while many resistant strains showed increased production

of an antagonist identified as PAB, other mechanisms of resistance also were involved.

Sulfonamide antagonists in filtrates of full-grown cultures of strains NRRL-313, 33, 38, and 67, of strains A, B, and C from the serial transfers, and of a sample of strains from each class of mutants isolated from plates were tested by the following methods: (1) ability of filtrates to antagonize NaST, thereby allowing growth of sensitive staphylococci; (2) ability of filtrates to replace PAB as a growth factor for *Acetobacter suboxydans* (LANDY and DICKEN, 1942); and (3) ability of filtrates to support growth of a *p*-aminobenzoicless strain of *Neurospora crassa*[4] (THOMPSON *et al.* 1943). Presence of PAB in filtrates of

TABLE 6

p-aminobenzoic acid production by sulfonamide-resistant mutants of Staphylococcus aureus.

MUTANTS FROM STRAINS WITH LOW PAB PRODUCTION				MUTANTS FROM STRAINS WITH INCREASED PAB PRODUCTION			
FROM NRRL-313, RESISTANT TO 1×10^{-3} M NaST		FROM STRAIN 33,** RESISTANT TO 5×10^{-4} M NaST		FROM STRAIN 38,*** RESISTANT TO 1×10^{-4} M NaST		FROM STRAIN 67,**** RESISTANT TO 1×10^{-3} M NaST	
MUTANTS TESTED		MUTANTS TESTED		MUTANTS TESTED		MUTANTS TESTED	
NaST RESISTANCE	INCREASE IN PAB PRODUCTION	NaST RESISTANCE	INCREASE IN PAB PRODUCTION	NaST RESISTANCE	INCREASE IN PAB PRODUCTION	NaST RESISTANCE	INCREASE IN PAB PRODUCTION
$5\text{–}6\times10^{-5}$	–*	$1\text{–}2\times10^{-4}$	–	$3\text{–}4\times10^{-4}$	–	2×10^{-3}	–
$1\text{–}2\times10^{-4}$	+	$5\text{–}6\times10^{-4}$	+	$5\text{–}6\times10^{-4}$	–	3×10^{-3}	–
$3\text{–}4\times10^{-4}$	+	$9\text{–}10\times10^{-4}$	+	$9\text{–}10\times10^{-4}$	–	4×10^{-3}	–

* –Indicates no increase over parent strain; +indicates increase over parent strain. Increased PAB production was by the same amount whenever it occurred.

** Colony isolation from NRRL-313 with same PAB production as strain NRRL-313.

*** Colony isolation from NRRL-313 with increased PAB production.

**** Colony isolation from strain 38 with same PAB production as strain 38.

S. aureus cultures is indicated by the fact that positive results were obtained with all assay procedures used.

A summary of the data on PAB production by different strains is given in table 6. Some resistant strains synthesized more PAB than the sensitive NRRL-313 strain; others did not. Mutants from NRRL-313 that were resistant to $5\text{–}6\times10^{-5}$ M NaST showed no increase in antagonist production; those resistant to NaST concentrations of 1×10^{-4} M and higher produced more antagonists than the parent strain. When strain 33 was used as the parent strain, mutants resistant to 1×10^{-4} M NaST showed no increased production of antagonist; mutants resistant to NaST molarities of 5×10^{-4} or higher showed increased antagonist production. Strains 38 and 67 already had undergone one mutation that resulted in formation of higher amounts of

[4] This strain of Neurospora was kindly supplied by DR. E. L. TATUM of YALE UNIVERSITY, New Haven, Connecticut.

PAB. None of the mutants from these strains showed further increases in production of antagonist. Mutants with increased PAB production fall into two classes when classified on the basis of NaST tolerance: those mutants with resistance increased by 10–20 times and those with resistance increased by 30–40 times. Increase in PAB production always was by the same amount. Therefore, while these mutations differed in effect on NaST tolerance, they had a common effect on PAB production.

All increases in production of sulfonamide antagonists were by a factor of ten times as measured by antagonism of NaST for sensitive *Staphhylococcus aureus*, by a factor of twenty times as measured by *Acetobacter suboxydans*, and by a factor of 30 times as measured by *p*-aminobenzoicless *Neurospora crassa*. An increase of 10–30 times in PAB production explained changes in sulfonamide tolerance of similar magnitudes when mutations affected both NaST resistance and PAB synthesis simultaneously. While it was a factor, production of antagonist did not account for the increases in resistance observed in all mutant strains, because mutants with increased NaST resistance and with no increase in PAB production were obtained from strain 38 and strain 67 (table 6). Also, strains with NaST resistance ten times that of NRRL-313 could be obtained by two successive mutations that did not affect PAB production.

Conclusions based on filtrates may be misleading. The use of both filtrates and culture extracts (MACLEOD, 1940) offers another approach, but it is questionable whether or not extracts correctly represent conditions in living cells. Also, it is difficult to determine if measurement of PAB in fully grown cultures gives an accurate estimate of conditions existent during the early stages of growth.

DISCUSSION

Discrete stepwise increases in resistance that occur at random time during serial transfer in NaST, and significant variation in the number of resistant cells in independent cultures, indicate mutation and selection as the mechanisms by which sulfonamide-resistant strains of *Staphylococcus aureus* originate. The same conclusion has been reached concerning resistance of *Escherichia coli* to bacteriophage (LURIA and DELBRÜCK (1943), resistance of *Staphylococcus aureus* to penicillin (DEMEREC 1945), and resistance of *Escherichia coli* to ultraviolet radiation (WITKIN 1946). Mutation and selection therefore appear to be the mechanisms by which resistance to antibacterial agents originates in many species of bacteria. In like manner, development of sulfonamide resistance in most species of microorganisms probably occurs by the same mechanisms as in *Staphylococcus aureus*.

SESLER and SCHMIDT (1942) reported experiments on serial transfer of pneumococci in sulfonamides. The data published showed stepwise increases in resistance that occurred at random time during transfer, but they concluded that the mechanism involved was one of adaptation, and that the steps were forced by the concentrations of sulfonamides used. Nevertheless, in the nine transfer series SESLER and SCHMIDT reported, there were seven instances where increases in resistance bypassed one and often two intervening concentrations of sulfonamide at one transfer. Also, adaptation does not explain the random-

ness in time of occurrence of the rises. Identical results obtained in the transfer experiments reported here were interpreted as constituting evidence for origin of resistance through mutation and selection.

Tests of the hypothesis that resistant bacteria arise by mutation should be made with organisms that have been grown in absence of inhibiting agents. This was impossible with sulfonamides because of inoculum-size effects. Nevertheless, validity of the data for determining the importance of mutations was not affected by the techniques used, for many cultures contained no resistant cells. This is conclusive evidence that physiological adaptation was not the mechanism by which resistant bacteria were developed. Preliminary exposure to sulfonamides could result in a serious error because of differential selection if several discrete mutations were involved at each step, for statistical analyses of the number of resistant cells in different cultures would be biased if the more resistant mutants had higher selective values. Clone size would reflect different resistance levels rather than random occurrence of mutations. The data gave no indication that such a condition existed, for highly significant variation between cultures was found in experiments where all mutants isolated showed identical resistance. Also, in some cases the highest number of colonies per culture was associated with low resistance.

Mutation rates were calculated from the percentage of cultures containing no resistant cells. Computation of mutation rates from the actual number of mutants present in the cultures could not be made from these data because of selection pressures existent during preliminary exposure to NaST.

The majority of mutants from NRRL-313 had NaST tolerance increased by about ten times, a few had NaST resistance increased by 30–40 times, and a few had NaST resistance increased by five times (table 1). All mutants with NaST tolerance increased by ten or by 30–40 times showed increased production of a sulfonamide antagonist capable of replacing *p*-aminobenzoic acid as a growth factor for *Acetobacter suboxydans* and for a *p*-aminobenzoicless strain of *Neurospora crassa*. Strains already resistant to ten times as much NaST as the parent strain NRRL-313 gave mutants with resistance increased by a second factor of ten times. Antagonist production was not altered by the second mutation. Therefore, some colony isolations from NRRL-313 might be expected to show an increase in resistance of ten times and no increased antagonist production. Such strains were not found. Either an insufficient number of colonies was tested, or the action of the genes concerned is conditioned by the genotype in which they occur. For example, mutations that increased sulfonamide tolerance by five times when they occurred in NRRL-313 may have given increases of ten times in strain 38. Another possibility is that mutations in strain 38 affect metabolic systems that are of importance in sulfonamide resistance only when PAB metabolism has been altered.

Several mechanisms of sulfonamide action also are suggested by the fact that the antagonist titer of filtrates depends on the method of assay used. Tests with *Staphylococcus aureus*, *Acetobacter suboxydans*, and *Neurospora crassa* each gave different results. NaST resistance of the parent strain NRRL-313 probably is the summation of several effects. Therefore, variation in PAB pro-

duction by a factor of 30 times as measured by Neurospora could show an increase of only ten times as measured by Staphylococci because some antagonists in the filtrate of NRRL-313 are effective in counteracting NaST but do not replace PAB as a growth factor for *p*-aminobenzoicless *N. crassa*. Antagonists other than PAB that may be present in the filtrate of NRRL-313 were not increased during the change from sensitivity to resistance, because tests with *S. aureus* gave the same anti-sulfonamide value for filtrates from all cultures with increased antagonist production.

Selection of resistant mutants probably is the primary factor responsible for development of drug-resistant strains that frequently occurs when a low dosage of sulfonamide is used either for prophylaxis or for long courses of treatment (EPIDEMIOLOGY UNIT NO. 22, 1945; BERNHEIMER 1946). Single-step mutants with high resistance occur at a low rate; for none were isolated either by DEMEREC (1945) or during this investigation. Bacteria with low drug tolerance were found to be relatively common, and if established as a strain, subsequently gave mutants with higher resistance. Therefore, if therapeutic doses are large enough to inhibit the primary mutants that may occur, development of highly resistant bacterial strains through selection of secondary mutants will be prevented. Treatments should be as intensive as possible in order to preclude development of resistant strains of bacteria.

SUMMARY

The mode of origin of sulfonamide-resistant strains of *Staphylococcus aureus* was studied by means of serial transfer in the presence of sodium sulfathiazole and by isolation of colonies from plates containing sodium sulfathiazole.

Stepwise rises in resistance that occurred at random time during serial transfer, and significant variation between similar cultures in the number of resistant cells they contained, indicated mutation and selection as the mechanisms by which resistant strains of bacteria were developed.

Mutant strains with moderate resistance gave rise to variants with still higher sulfonamide tolerance. Bacteria capable of growing in saturated solutions of sodium sulfathiazole were obtained by successive mutations to resistance.

Calculations based on the proportion of cultures that failed to show resistant cells gave rates of mutation from sensitivity to resistance that ranged from 2 times 10^{-9} to 4 times 10^{-10} per bacterium per generation.

At least five different mutations affect the sulfonamide resistance of *S. aureus*. Only one or possibly two mutations are associated with increased production of *p*-aminobenzoic acid. Therefore, it appears as if several cell mechanisms are sensitive to sulfonamides and are altered by mutations.

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STUDIES ON THE INHERITANCE OF APOMIXIS AND STERILITY IN THE PROGENY OF TWO HYBRID PLANTS IN THE GENUS *PARTHENIUM*

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THE inheritance of the method of reproduction in guayule and mariola (*Parthenium argentatum* Gray and *P. incanum* H. B. K.) is of practical significance in the breeding program and has interesting theoretical implications. Facultative apomixis has been shown to be the predominant method of reproduction in these two species, although sexual reproduction occurs in both (POWERS and ROLLINS 1945, ROLLINS 1945, ESAU 1944). All strains of Guayule studied thus far with 54± or more chromosomes reproduce by facultative apomixis. All known 36+ chromosome strains of guayule reproduce sexually. Mariola has not been studied as extensively as guayule but one sexual member of this species has been identified (POWERS and ROLLINS 1945). This plant has not been studied cytologically. The descendants of one hybrid plant with high chromosome numbers have been shown to reproduce sexually (GARDNER 1946a). Intermediate cases involving fertilization without reduction have been described by POWERS (1945).

The present study has shown the degree of variation in measurable characters expressed by individual members of different progenies to be inherited in quantitative fashion. Data obtained from controlled and open pollinated crosses are used in determining the extent to which sexual processes of reduction and fertilization are occurring. Plants resulting from complete sexual reproduction are expected to differ markedly from each other. Those originating by nonsegregating apomixis should not differ significantly if the environment is controlled. Intermediate cases would be expected to show gradations in variation between the two extremes depending on the extent to which sexual processes are occurring. The studies reported here demonstrate the facultative nature of the method of reproduction and show that the extent to which normal sexuality occurs in a strain is controlled at least in part by genetic factors.

MATERIALS AND METHODS

Material used in these studies was selected from the field plantings of guayule and mariola at Salinas, California. These plantings were grown from the seed collections of POWERS in Mexico and POWERS and FEDERER in Texas. All crosses described in this paper were made in the field at Salinas, California. Seed parent plants for the controlled crosses and open pollinated progeny were chosen from two different individual plant selections (accessions 42418 and 4253). The first crosses were made during the summer of 1944. Emasculation was accomplished by removing the young disc florets from each isolated flower

head before any ray florets were open. When the ray florets reached the receptive stage pollen from another individual isolated plant was applied to the stigma with a camel hair brush. Applications of pollen were made on successive days until the stigma had dried. Groups of flowerheads thus pollinated were isolated with small percale bags. The seed was ripened in the bags, picked, treated by the method described by BENEDICT and ROBINSON (in press), and germinated between wet blotters in a germinator. The seedlings were transferred to pots and raised in sterile sand culture being nourished with Hoagland's solution (HOAGLAND and ARNON 1939). The young plants were transplanted to the field in the spring of 1945. Individual plants from the various progenies have been selfed, backcrossed to both parental strains, and outcrossed in various combinations. Progeny resulting from these later crosses have also been studied and compared.

Some chromosome determinations were made by DR. A. D. BERGNER (1944) from pollen-mother-cells. Others were made by the writer from aceto-orcein root tip smears prepared according to MEYER'S (1945) technique. Since aneuploidy is common in these plants a slight variation in chromosome number was often observed in different members of a group. The "±" given with chromosome determinations of polyploids indicates that deviations of one or two chromosomes in either direction have been observed or might be expected. No 2n chromosome numbers lower than 36 have been found. The + following the determination for diploids indicates that one or two more than 36 have been observed or might be expected.

Two hybrid plants from selections listed above and their progeny were used for the crosses. Both expressed a wide variety of guayule and mariola characters useful for purposes of classification and comparisons of variation in the progeny. Preliminary observations concerning one of these hybrids (42418) and its immediate progeny (culture 3053) suggested a high degree of sexuality. The other hybrid (22-7) arose from a natural cross between a sexual 36+ chromosome guayule plant and a mariola plant and proved to be partially apomictic. Data obtained from crosses involving these two hybrids, differing widely in chromosome number, tendency toward self sterility, and method of reproduction, are used in analyzing problems concerning the inheritance of apomixis and sterility

42418 and its Progeny

The maternal parent (accession 42418) of plants used in the first series of crosses was described at the Wyatt Ranch, Texas, by POWERS (1942) as being intermediate between guayule and mariola. Seed was collected on an individual basis so that its hybrid nature might be determined. ROLLINS (1944) suggested that the plant must have been a natural hybrid between the two species. Twenty-three plants were grown from the seed of this hybrid and placed in the field in the spring of 1943 as culture 3053. The plants were described and their sexual nature was discussed in a previous paper (1946a). Characteristics of both guayule and mariola were obvious. Chromosome counts

showed the plants to have chromosome numbers, ranging from 70 to 95. ROLLINS (personal correspondence) has plants originating from the same seed selection which have more than 100 chromosomes. These hybrids do not show the marked aberrancy described by POWERS (1945) and POWERS and ROLLINS (1945) in guayule strains with high chromosome numbers. This might be expected from hybrids favoring mariola, as these do, since ROLLINS (1945) has observed that a high chromosome number is tolerated without aberrant effects in the progeny of mariola seed parents.

Three members of the group (3053-10, 14, 19) were chosen as seed parents for controlled crosses. These three seemed to represent the spread of characteristics of the group. The plant identified as 3053-10 was more like guayule than the others, 3053-14 strongly favored mariola, and 3053-19 was more nearly intermediate between guayule and mariola, although it favored mariola in many characteristics. Since the three plants chosen were large plants, several crosses could be made on each plant at one time. Each plant was selfed, crossed with a member of strain 593 (a commercial strain of guayule developed by DR. W. B. MCCALLUM), a 36+ chromosome sexual guayule strain (accession 4255) from Durango, Mexico, and mariola (accession 43684).

In addition to the controlled crosses described above a progeny test was conducted to compare the open pollinated progeny of all members of culture 3053. The experimental design was a randomized complete block with ten replications and 100 seeds per replicate. A total of 1000 seeds were sown for each entry. Seed from accession 4263, a 54± chromosome, apomictic, strain from Durango, Mexico, and strain 593, demonstrated by POWERS and ROLLINS (1945) to be 91.3 percent apomictic, were included as checks. The percentage germination was low and a good many seedlings were lost from damping off. By the time the transfer to the field was made the number of living plants from five of the 23 parent plants was lower than 20. These five were not included in the field planting. The progeny of the other eighteen plants and the two checks were rerandomized. All operations and measurements in the field were performed on the basis of replicates to control any differences which might occur through environmental agencies. The coefficient of variability is used to express the degree of variation methematically and provide an expression suitable for comparing the variation expressed in the different sets of data.

The characters studied include: height of plant, spread of plant, length of leaf, length of petiole, width of leaf and number of points on leaf. The height is the vertical distance from the ground to the top of the highest leaf on the plant. The spread is the distance from one side to the other through the center of the plant. Leaf measurements were obtained by picking ten leaves at random from each plant and measuring the length and width of the leaf and length of the petiole. The leaf length measurement represents the distance from the tip to the point of attachment of the petiole with the plant stem. The width is measured just above the first two major points. The petiole is measured from the attachment end to the place where the blade of the leaf widens abruptly. The number of points represents the total number of points

on the leaf and petiole not including the tip of the extremity of the leaf.

During the summer of 1945 several of the plants resulting from open and controlled pollination of members of culture 3053 were selfed. Since other studies by the writer (1946b) had shown that some types of cloth bags are not effective as isolators against guayule pollen kraft paper bags were used along with the percale bags as checks. Results obtained from selfing with the two kinds of isolators were comparable (table 4) giving assurance that the cloth used in these experiments was an effective barrier against guayule pollen from other plants. The number of seeds obtained from each selfed plant ranged from 200 to 1508. The seeds were treated and germinated as described above. The young plants representing the third generation from the hybrid growing in Texas have been studied and compared.

22-7 and its Progeny

The second series of crosses involved another guayule-mariola hybrid (22-7) and its progeny. Its maternal parent was an individual plant (accession 4253) from Durango, Mexico. Most plants grown from this seed selection were found to be sexual, self sterile, and to have 36+ chromosomes. The plant (22-7) chosen for the crosses reported here showed characteristics of both guayule and mariola, was found to have 54± chromosomes, and was suspected of being partially apomictic. Obviously this plant resulted from a natural cross between the guayule plant from which POWERS collected seed and a nearby mariola plant. Crosses were made between 22-7 and a 36+ chromosome strain (accession 4255). The plant was selfed and open pollinated seed was also secured. The seed resulting from these three treatments was prepared and germinated as described above and the young plants were placed in the field. The same six characters described above and one additional character, pollen grain size, were measured and the data were calculated and analyzed. The pollen grain size was determined by measuring 100 pollen grains from each plant and calculating the average size in microns. The technique used in preparing the pollen grains for microscopic examination is given in another paper (POWERS and GARDNER 1945).

EXPERIMENTAL RESULTS AND DISCUSSION

Progeny of 42418

In order to illustrate the variation observed among individual plants from each progeny six characters were measured and the results were analyzed mathematically. Data obtained from the controlled crosses involving members of culture 3053 are given in tables 1 and 2. To save space the results from crosses involving only one seed parent (3053-19) are given. Comparable results were obtained from crosses involving the other two seed parents with which parallel crosses were made. Only the means and coefficients of variability of the characters measured are given. Since the same seed parent was used in all crosses and the environmental factors were controlled the data are directly comparable. Data concerning the strains from which the pollen parents were

chosen are also given for comparison. An examination of the means (table 1) will show that most progeny are intermediate between the two parent strains in height and spread and leaf size. Some progeny, particularly those resulting from the cross between 3053-19 and strain 593 are comparable in size or even

TABLE 1

Means and their standard errors for designated plant characters in progeny of crosses involving 3053-19. Data for the two parental strains of guayule and one of mariola are given for comparison.

	MEAN AND STANDARD ERROR*						
	3053-19 SELFED	GUAYULE (593)	3053-19× 593	MARIOLA	3053-19× MARIOLA	GUAYULE (4255)	3053-19× 4255
Height of plant	25.6 ± .51	35.9 ± .33	23.4 ±2.9	71.9 ± .57	33.3 ±2.3	38.3 ± .51	29.3 ±1.0
Spread of plant	35.1 ± .73	52.5 ± .59	29.4 ±4.2	100.7 ± .72	47.9 ±2.9	68.4 ±1.1	40.3 ±1.3
Length of leaf	4.6 ± .29	5.5 ± .08	4.2 ± .18	4.0 ± .10	4.2 ± .12	5.1 ± .08	5.3 ± .28
Length of petiole	1.3 ± .12	2.3 ± .05	1.4 ± .08	.64± .02	.83± .07	1.6 ± .05	1.7 ± .09
Width of leaf	.66± .06	.75± .01	.67± .04	.65± .02	.64± .02	.91± .02	.73± .08
Points on leaf	9.6 ±1.3	1.9 ± .06	4.6 ± .27	12.7 ± .34	11.5 ± .99	1.2 ± .12	6.2 ± .78

* Height of plant, spread of plant, length of leaf, length of petiole and width of leaf are given in centimeters. Points on leaf represents the total number of points not including the tip at the extremity of the leaf.

TABLE 2

Coefficients of variability and their standard errors for designated plant characters in progeny of crosses involving 3053-19. Data for the two parental strains of guayule and one of mariola are given for comparison.

	COEFFICIENT OF VARIABILITY AND STANDARD ERROR						
	3053-19 SELFED	GUAYULE (593)	3053-19× 593	MARIOLA	3053-19× MARIOLA	GUAYULE (4255)	3053-19× 4255
Height of plant	26.5± 1.5	6.5± .65	39.2±10.0	4.0± .56	27.9±5.3	12.8± .95	23.9± 2.5
Spread of plant	28.7± 1.6	7.8± .78	44.9±11.9	3.5± .50	24.4±4.6	15.9±1.2	23.0± 2.4
Length of leaf	19.5± 4.6	4.8±1.1	14.0± 3.2	8.0±1.8	8.9±2.0	11.1±1.2	16.6± 3.8
Length of petiole	28.8± 6.9	6.3±1.4	18.4± 4.3	9.5±2.1	26.9±6.4	21.9±2.4	17.1± 3.9
Width of leaf	27.5± 6.6	4.2± .94	19.5± 4.5	10.3±2.3	13.2±2.9	18.0±1.9	34.4± 8.5
Points on leaf	43.6±11.6	9.6±2.1	18.2± 4.2	8.4±1.8	27.5±6.6	66.3±9.1	40.0±10.2

smaller than the smaller parent (3053-19) selfed. Hybrid vigor was likely expressed in the seed plant (3053-19) which was one of the largest plants in the field. Progeny from selfing and crossing have apparently lost the hybrid vigor and suffered an additional loss, reducing the size in some cases to a level lower than that of the parent strains. This latter loss is likely the result of interference in development of the plants caused by the high chromosome number. An unsatisfactory genic balance has evidently accompanied the piling up of chromosomes.

An examination of the coefficients of variability (table 2) will show that the progeny of 3053-19 selfed are highly variable in all six characters studied. Plants from the apomictic commercial strain of guayule (strain 593) show com-

paratively little variation in the six characters. Coefficients obtained for the progeny of the cross between 3053-19 and 593 are large. The strain of maricla, known to be highly apomictic (POWERS and ROLLINS 1945), shows little variation, but the progeny of the cross between 3053-19 and mariola vary strikingly. The 36+ chromosome sexual strain of guayule (accession 4255) shows marked variation but the coefficients are lower than those for 3053-19 selfed in all characters except points on leaf. The progeny of the cross between 3053-19 and 4255 also show high coefficients of variability. All progeny from

TABLE 3

Coefficients of variability for designated characters in open pollinated progeny of various plants in culture 3053.

	HEIGHT	SPREAD	LENGTH OF LEAF	LENGTH OF PETIOLE	WIDTH OF LEAF	POINTS ON LEAF
593	6.472	7.845	4.862	6.327	4.237	9.646
4263	15.608	16.802	13.662	13.220	9.161	no points
3053-2	15.546	19.758	9.854	10.955	7.656	10.210
3053-18	17.674	24.794	8.687	17.732	17.414	27.058
3053-1	24.190	28.788	16.793	29.308	15.438	24.345
3053-7	21.241	23.397	14.215	20.947	22.450	33.866
3053-6	28.083	31.320	5.271	24.141	15.378	65.445
3053-24	28.859	35.808	9.751	20.758	16.617	41.039
3053-20	20.893	32.980	17.707	31.498	15.495	27.284
3053-5	32.827	41.616	13.976	23.030	15.400	37.716
3053-22	33.401	24.856	16.583	13.642	16.914	68.799
3053-11	31.209	36.687	23.391	30.165	18.069	15.906
3053-19	32.906	33.806	—	—	—	—
3053-14	25.236	43.490	23.206	27.264	21.575	32.770
3053-13	46.122	43.129	16.136	16.521	24.552	40.206
3053-10	36.968	43.691	22.848	19.534	21.317	45.505
3053-23	42.252	42.835	19.397	22.980	32.738	33.609
3053-21	33.311	34.100	18.438	26.757	25.820	48.152
3053-4	28.556	39.606	19.542	35.072	23.342	59.974
3053-3	64.465	47.412	23.313	34.039	22.192	43.590

the sexual parent have high coefficients for most characters. This suggests that sexuality is well established in 3053-19 and that the factors controlling the major sexual processes are dominant.

The open pollinated progeny of the members of culture 3053 have also been studied and compared in the field. Much variation has been observed in size, color, and general appearance of the plants within the plots as well as between the plots. Several people while walking through the field have commented that no two plants are alike. No other place in the entire experimental field of more than 100 acres is marked by such striking variation among individual plants. In contrast, the plots containing the apomictic plants used as checks appear strikingly uniform.

The same six characters mentioned above were measured in the open pol-

linated progeny. The data are summarized in table 3. In the interest of space only the coefficients of variability are given. Plants from which the seed was picked are identified on the left hand side of the table. All except the first two entries are members of culture 3053. The two checks (strain 593 and accession 4263) appear at the top of the table. The low coefficients of variability shown for strain 593 are expected since this strain is highly apomictic. The two year old plants representing accession 4263 showed marked uniformity. Controlled pollinated seed gave uniform plants like the parents. The present data show the second generation progeny resulting from open pollination to be more variable than 593 but less variable than all of the progeny from members of culture 3053 with the possible exception of 3053-2.

In the table the plants are arranged roughly in order of their variability. Most groups showing a high coefficient of variability for one character show a high coefficient for other characters also. On the whole the coefficients of variability are slightly higher than those from controlled crosses (table 2). This can be explained by the difference in pollen source. All progeny arising from a controlled cross received pollen from the same plant and therefore had both seed and pollen parents in common. The variation would depend on segregation and recombination of factors present in the two parents. The open pollinated plants receiving pollen brought to them by natural agents (see GARDNER 1946b) from the wide variety of plants in the experimental plots would be expected to show more variation among individual offspring. Since all members of culture 3053 were equally accessible to pollen from neighboring plants the source would not be expected to influence the relative degree of variability observed among progenies of different plants.

A gradation (table 3) extends over a rather wide range from the progeny of 3053-2, with coefficients in the same order of magnitude as 4263, to the progeny of 3053-3 with considerably higher coefficients. The three members of the culture (3053-10, 14, 19) which were used in controlled crosses appear in the same order in both sets of data. Since the plot containing culture 3053 is entirely surrounded by apomictic plants it is likely that most of the open pollinated progeny received pollen from apomictic plants. The fact that all expressed a fairly high degree of sexuality which persisted through two succeeding generations (see below) suggests again that the major factors determining sexuality are dominant. The gradation observed suggests a segregation of several modifying factors which influence major processes differentiating reduction and nonreduction and possibly also fertilization and nonfertilization. This segregation would result in different degrees of sexuality in different plants. The data indicate that when the major processes are established the degree of sexuality is inherited quantitatively. This segregation of modifying factors may account for the facultative nature of the method of reproduction in guayule and mariola. Chromosome counts (GARDNER 1946a) obtained for several plants from controlled crosses show expected chromosome numbers on the basis of complete reduction and fertilization. The differences occurring in these plants, therefore, must be genic and not chromosomal. Progeny showing high coefficients (3053-3) may be assumed to have arisen largely as a result

of normal sexuality (reduction followed by fertilization). Those showing lower coefficients (3053-2) may have received from their parents chance accumulations of modifying factors favoring pseudogamy and restricting reduction. It must be pointed out that in spite of the gradation observed most of these groups show marked variation, indicating a high degree of sexuality.

There seems to be no correlation between the coefficients of variability for characters analyzed and the numbers of progeny obtained from the same plants. This indicates that the segregating factors which control variability and those influencing compatability and viability in these plants are independent. A correlation has been observed in another case described below.

Several plants resulting from crosses described above were selfed, thus producing a third generation progeny from the original plant in Texas (accession 42418). These plants show the same high degree of variation recorded for culture 3053 (GARDNER 1946a) and for the progeny of culture 3053 described above. The germination of seed (table 4) from most selfed plants was extremely low, but wide variation was observed. The highest percentage recorded (14.5 percent) was for the progeny of 6505-2, a plant arising from a cross between 3053-19 and mariola. This plant also produced a greater number of seeds (1508) than any other plant selfed. Other plants arising from the same cross produced a lower percentage germination ranging from 4.41 percent for 6505-1 to .72 percent for 6050-11. Wide variation was observed in the germination of seed from most of the plants selfed.

On the whole the percentage germination is considerably lower for the selfed plants than for the progeny of the original crosses (also given in table 4). This suggests a high degree of self incompatibility. Incompatibility factors have apparently come to expression in these selfed plants and accounted, at least in part, for the low germination. Some strains of guayule, particularly the diploid strains, are known to be highly self sterile. Members of culture 3053 which have been selfed show a percentage germination ranging from 7.2 for 3053-19 to 3.4 for 3053-14. The second generation of selfed plants is considerably lower in percentage germination than the first. This suggests that sterility factors have become homozygous by continued selfing and have come to expression. Some of these factors undoubtedly influence the rate of pollen germination and formation of the pollen tube (see below). Where an interspecific cross is involved there are probably many sources of incompatibility and it is difficult to determine the extent to which inherited sterility factors are involved. The data, however, suggest a segregation of recessive sterility factors which are quantitative in action.

Progeny of 22-7

The progeny resulting from crosses and selfs involving 22-7 were classified by inspection in the field when they were one year old. Two classes were readily distinguished. Some plants were observed to resemble closely the hybrid seed parent. They were compact like 22-7 and uniform. The leaves were strikingly similar to those of the mother plant. Other plants appeared more bushy and showed wide variation among themselves. They resembled guayule plants

TABLE 4

Energence data from selfed progeny of field crosses involving members of culture 3053.

PLANT SELFED	PARENTAL CROSS		PERCALE ISOLATOR	"KRAFT" PAPER ISOLATOR	NO ISOLATOR
	PLANTS CROSSED*	PERCENTAGE GERMINATION	PERCENTAGE GERMINATION	PERCENTAGE GERMINATION	PERCENTAGE GERMINATION
6501-4	3053-19×42354	6.5	.50		
6501-6	3053-19×42354		10.58		
6501-8	3053-19×42354		.32		
6501-17	3053-19×42354		3.67	3.45	
6501-18	3053-19×42354		11.45		
6502-1	3053-14×42354	2.	4.36		
6502-2	3053-14×42354		.41		
6503-1	3053-19×593 V	7.	4.16		
6503-2	3053-19×593 V		.69		
6503-6	3053-19×593 V		.16		
6503-9	3053-19×593 V		.78		
6503-11	3053-19×593 V		—		
6505-1	3053-19×43684	11.	4.41		
6505-2	3053-19×43684		14.59		
6505-9	3053-19×43684		.88		
6505-11	3053-19×43684		.72		
6506-1	3053-14×43684	18.0	.62	.69	
6506-4	3053-14×43684		.41		
6507-5	3053-19×4255	12.0	.89	1.20	
6507-6	3053-19×4255		.69	.61	
6507-8	3053-19×4255		.41	.46	
6507-10	3053-19×4255		.47	.47	
6507-17	3053-19×4255		1.14	.71	
6507-30	3053-19×4255		.25	—	
6507-43	3053-19×4255		1.43	.35	
6508-1	3053-14×4255	12.0	.27	.94	
6508-6	3053-14×4255		—		
6508-7	3053-14×4255		2.22		
6509-9	3053-19 selfed	7.2	.31	—	
6509-58	3053-19 selfed		.31	—	
6510-1	3053-14 selfed	3.4	1.05	—	
6510-5	3053-14 selfed		1.05	2.22	
6510-7	3053-14 selfed		.89	—	
Strain 593	Open pollinated				7.00
44252-66	4255 selfed		.50		29.40
44252-107	4255 selfed		—		26.57
44253-2	4255 open pollinated		.90	2.00	24.00
44253-34	4255 open pollinated		1.6		29.00

* Seed parents are always written first.

more closely than mariola and many of them bore a strong resemblance to the 36+ chromosome strain from which the pollen for the cross was secured. Most of the plants belonging to this group were larger than those resembling the seed parent but considerable variation was observed in size. The classifi-

TABLE 5

Means and their standard errors for designated plant characters in the progeny of crosses involving 22-7. Data for the strain of guayule (4255) from which the pollen parent was chosen are also given for comparison.

	MEAN AND STANDARD ERROR*					
	APOMICTS OPEN POLLINATION	AMPHIMICTS OPEN POLLINATION	APOMICTS 22-7×4255	AMPHIMICTS 22-7×4255	APOMICTS 22-7 SELFED	GUAYULE SEXUAL STRAIN (4255)
Height of plants	35.7 ± .35	39.0 ± .98	34.4 ± .36	39.6 ± .77	36.3 ± .79	38.3 ± .51
Spread of plants	52.7 ± .38	57.4 ± 1.5	51.2 ± .50	54.3 ± 1.0	54.7 ± .69	68.4 ± 1.1
Length of leaf	4.5 ± .08	5.6 ± .28	4.3 ± .08	5.4 ± .28	4.6 ± .05	5.1 ± .08
Length of petiole	1.1 ± .02	1.7 ± .12	.98± .02	1.5 ± .06	1.1 ± .02	1.6 ± .05
Width of leaf	.57± .006	.85± .06	.56± .01	.76± .06	.56± .006	.91± .02
Points on leaf	7.7 ± .17	5.2 ± .35	7.7 ± .12	5.3 ± .24	7.5 ± .15	1.2 ± .12
Size of pollen	24.89± .148	27.27± .455	25.25± .132	27.82± .371	25.16± .065	—

* Height of plant, spread of plant, length of leaf, length of petiole, and width of leaf are given in centimeters. Points on leaf represents the total number not including the tip at the extremity of the leaf. Size of pollen grains is given in microns.

TABLE 6

Coefficients of variability and their standard errors for designated plant characters in the progeny of crosses involving 22-7. Data for one sexual guayule strain (4255) and one apomictic guayule strain (593) are given for comparison.

	COEFFICIENT OF VARIABILITY AND STANDARD ERROR						
	APOMICTS OPEN POLLINATION	AMPHIMICTS OPEN POLLINATION	APOMICTS 22-7×4255	AMPHIMICTS 22-7×4255	APOMICTS 22-7 SELFED	GUAYULE SEXUAL STRAIN (4255)	GUAYULE APOMICTIC STRAIN (593)
Height of plants	6.3± .70	14.9±1.8	8.6± .89	13.9±1.4	8.5±1.5	12.8± .95	6.5± .65
Spread of plants	4.5± .51	16.0±1.7	6.6± .69	13.6±1.4	4.9± .90	15.9±1.2	7.8± .78
Length of leaf	5.6±1.3	15.6±3.6	6.0±1.3	16.8±3.9	3.8± .85	11.2±1.2	4.9±1.08
Length of petiole	5.5±1.2	23.3±5.5	7.6±1.7	13.5±3.06	6.9±1.5	21.9±2.5	6.3±1.4
Width of leaf	3.5± .79	22.4±5.3	6.4±1.4	25.4±6.5	3.4± .76	18.0±1.9	4.2± .94
Points on leaf	6.9±1.5	21.3±4.9	4.8±1.1	12.6±2.6	6.5±1.5	66.3±9.1	9.6±2.1

cation was checked by other investigators familiar with the characteristics of these plants and perfect agreement was obtained. The two distinct classes were also distinguished on the basis of measurements (tables 5 and 6) and progeny tests (described below).

The progeny resulting from the selfs were all like the mother plant. The number of progeny was small. From more than 500 seeds only 15 plants developed. This indicates that the plants are not capable of self fertilization but

a comparatively few eggs are capable of pseudogamous development. The fact that pollen is required for the development of the egg was demonstrated by emasculating large numbers of flowers from the same plant. These flowers were isolated in the same manner as those being selfed and crossed. None of the seeds germinated, showing that pollen is required for the activation of the egg.

A total of 98 plants was obtained from the cross between 22-7 and the sexual plant (accession 4255). Of these, 46 were like the mother (apomictic) and 52 were distinctly different. The plants resembling the seed parent were identical with those resulting from selfing. Obviously these plants were produced by nonsegregating apomixis (without reduction or fertilization). The small degree of genetic variation which was revealed by careful measurements (table 6) while the environmental factors were controlled can be explained on the basis of limited segregation which has been shown to occur in agamic complexes (GUSTAFSSON 1935, 1939, 1946, STEBBINS 1941). Plants belonging to the other class showing greater variation and expressing characteristics of the pollen parent were obviously produced through fertilization (amphimixis). Since reduction did not take place in other eggs from the same plant it seems reasonable to assume that it did not occur here either. Evidence presented below supports this assumption. The factors accounting for the genetic variation observed (table 6) were introduced with the pollen.

A total of 75 plants were secured from the open pollinated seed. Of these, 40 were apomicts and 35 were amphimicts. There is no significant deviation from a one to one ratio between the two classes in the results of the cross or the open pollinated progeny. The plants in the same plot with 22-7 were 36+ chromosome sexual plants. It is likely that most of the pollen reaching 22-7 came from these plants which were closely related to those used as pollen parent in the cross described above. Some pollen may have come from plants from other strains at a greater distance. Since the ratio is the same for the controlled and open pollinated progeny the source of pollen had no effect in determining which unreduced eggs would be effectively fertilized. All open pollinated flowers were equally exposed to pollen from neighboring plants and all flowers involved in the cross were known to have received pollen from the same plant. Therefore, some factor in the female parts of the plant must determine whether or not each unreduced egg is fertilized.

MEYER (unpublished) has observed that when pollen of a highly self sterile 36+ chromosome plant was placed upon the stigma of the same plant very few pollen grains formed tubes that entered the stigmatic tissue and these few did not grow far into the tissue. After crossing there were usually many empty pollen grains attached to the stigmatic surface and many long pollen tubes deep in the stylar tissue. Some crosses, however, behaved essentially as selfs and showed a high degree of incompatibility. This has been interpreted to indicate that at least some incompatibility factors influence the rate of pollen tube growth. The variation in compatibility observed in different crosses (see also table 4) would suggest a segregation of these factors. The data presented here

show that pollen from 22-7 only very rarely succeeded in self fertilization. Pollination was insured by transferring pollen from the disc florets to the stigmas within the ray florets of the same plant. Pollen from other plants accomplished fertilization more readily. The same method of pollination was employed in the controlled crosses but pollen was transferred from another individual plant. Half the plants which developed were fertilized. Incompatibility factors may control the degree of attraction of the female parts of the flower for the pollen or the degree of resistance of the stigmatic tissue to pollen tube growth.

The first step in the transition from sexual diploids to facultatively apomictic polyploids (according to POWERS' (1945) hypothesis) would be the change from reduction to non-reduction. This step has apparently been accomplished in one generation in the material here described. The second step would be the elimination of fertilization. Chance accumulations of incompatibility factors concerned with the growth of the pollen tube might explain the mechanism by which this is accomplished. In the material described here this second step is in a transitional stage midway between the two extremes (apomixis and amphimixis). The third step involving the ability of unfertilized, unreduced eggs to develop has made its appearance in the small number of selfs and greater number of the progeny resulting from crosses which are capable of developing without effective fertilization.

In an attempt to illustrate accurately the variation observed among the amphimicts and make comparisons with the apomicts seven different characters were measured. These included the same six characters described above for 42418 and one additional character, the diameter of the pollen grains. The data are summarized in tables 5 and 6. Only the means and coefficients of variability are given. Comparison of the means will show that the amphimicts are consistently larger in height, spread, leaf length, petiole length, leaf width, and pollen grain size. The apomicts have a greater number of points on the leaves like the seed parent. The larger size of the pollen grains produced by the amphimicts suggests a difference in chromosome number. The fact that the diameter of the pollen grains in guayule is proportional to the number of chromosomes within the grain was established by parallel determinations of chromosome number and pollen grain size of many individual plants throughout the polyploid series (BERGNER 1946). By reference to POWERS' (1945) classification the apomicts would fit into the $54\pm$ chromosome group and the amphimicts would have $72\pm$ chromosomes. This substantiated the assumption that the apomicts have arisen from pseudogamous development of unreduced eggs. The amphimicts from controlled crosses must have arisen from unreduced ($54\pm$ chromosome) eggs fertilized by reduced (18 chromosome) pollen from the sexual pollen parent.

Comparison of the coefficients of variability (table 6) will show the amphimicts to be consistently more variable than the apomicts. The coefficients for most characters studied are lower for the amphimicts than those listed in table 2 for the progeny of a completely sexual plant. The case of 22-7 must be con-

sidered one of partial sexuality. Reduction does not occur to any appreciable extent in any of the progeny. The apomicts being pseudogamous are not influenced by the factors in the pollen. These factors do, however, enter into combination and express themselves in the amphimicts thus accounting for the greater variation observed.

Eighteen of the plants resulting from the crosses and selfs involving 22-7 were selfed. Approximately 500 seeds were obtained from each plant. An average of 12.1 percent germination was obtained for the amphimicts compared with 2.28 percent for the apomicts. This difference may be explained, at least in part, on the basis of segregation of factors for sexuality. Plants which by chance received factors preventing fertilization and produced eggs largely incapable of pseudogamous development would not produce many viable seeds. Established apomictic strains have apparently accumulated factors enabling them to develop as apomicts without fertilization. These partially sexual plants, only two generations removed from a completely sexual strain, would not be expected to have these factors well established. Unreduced eggs incapable of pseudogamous development would become lethals if they were not fertilized. In another case described above involving plants expressing a higher degree of sexuality no correlation was observed between degree of sexuality and numbers of progeny obtained. The reason may be that all eggs were equally capable of fertilization. Factors favoring complete sexuality particularly those concerned with fertilization were obviously better established in the other case mentioned involving accession 42418.

The progeny of the apomicts resulting from the cross between 22-7 and the sexual plant were practically all apomicts. A few amphimicts appeared indicating that self fertilization was accomplished in a few cases. The amphimicts resulting from the same original cross produced apomicts and amphimicts in the proportion of one to one like their seed parent. It might be pointed out here that the amphimicts when selfed produced the same two classes in the same proportions as the original plant (22-7) produced when crossed with the sexual plant. Factors introduced with the pollen in the previous generation have evidently increased the self compatibility and sexuality above the expected level. The apomicts resulting from the selfing of 22-7 produced all apomicts like their seed parent.

An example of the development of apomictic polyploids from a cross involving a sexual diploid plant is observed in the history of 22-7 and its progeny. The 54± chromosome partially sexual plant was produced from a natural cross between a 36+ chromosome, sexual, self sterile, guayule plant and a 72± chromosome mariola plant, presumably apomictic. Reduction did not occur and only half of the unreduced eggs which developed were capable of fertilization. These conditions would tend toward maintenance of the triploid stand. The half of the eggs which were capable of fertilization became tetraploids. This example lends support to the hypothesis developed by POWERS (1945) to explain the origin of apomixis and polyploidy. POWERS describes the mechanism by which such polyploids might have arisen originally and become established in nature.

SUMMARY AND CONCLUSIONS

Data obtained from studies involving the progenies of two guayule-mariola hybrids have been used in analyzing certain features of the inheritance of apomixis and sterility. The progeny of one hybrid (42418) were found to reproduce through sexual processes (reduction followed by fertilization). A gradation was observed in the degree of sexuality expressed by different members of the group. The data suggest that the major sexual processes are well established. The small differences observed can be explained on the basis of segregation of modifying factors. The origin of this sexual polyploid can be explained on the basis of a chance combination of major factors and modifiers favorable to sexuality.

The second hybrid (22-7) proved to be partially sexual. Reduction did not occur to any appreciable extent and fertilization was accomplished in only part of the progeny. Two distinct classes were thus produced. An example of the development of apomictic polyploids from a cross involving a sexual diploid has been observed in this material.

Two types of sterility were encountered. In one case incompatibility was observed in plants representing transitional stages between apomixis and amphimixis. These plants were incapable of fertilization but did not have the ability (common to established apomicts) to develop without fertilization. The other type was apparently controlled by recessive factors which were accumulated and came to expression through selfing of plants resulting from crosses involving self sterile strains. Some of these factors likely influence the development of the pollen tube.

The study has demonstrated the fact that sexual polyploids representing the genus *Parthenium* can occur in nature. None have been reported in guayule. One sexual plant not studied cytologically has been reported in mariola. The data suggest that the apomictic processes, failure of reduction, failure of fertilization and pseudogamous development are controlled independently. The major factors favoring sexual processes must be few in number and they appear to be dominant. Modifying factors are also postulated and may account to a large extent for the facultative nature of processes involved in reproduction in this group.

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A SPECTROPHOTOMETRIC ANALYSIS OF THE EYE COLORS OF HABROBRACON

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EXPERIMENTS using a variety of techniques, histological, genetic, transplantation and chemical, have shown that the normal wild type eye color of Drosophila is caused by two pigments, red and brown (JOHANNSEN, 1924; WRIGHT 1932; MAINX 1938; see EPHRUSSI and HEROLD 1944, for review). These pigments are chemically quite different, the end results of two chains of reactions in development which are separate but seem to have a common step (EPHRUSSI and HEROLD 1945). The red pigment is soluble in acid ethyl alcohol (AEA, 30 percent ethanol acidified to pH_2) and the brown pigment is soluble in acid methyl alcohol (AMA, absolute methyl alcohol acidified with 1 percent HCl). The eye color of the flour moth, Ephestia, also depends on the presence of red and brown pigments similar to, if not, identical with, those of Drosophila (BECKER 1939). In the blow-fly, Calliphora, however, BECKER (1942) found only a brown pigment, similar to but not identical with the brown pigment of Drosophila.

The existence of a large number of eye color mutants in the parasitic wasp, *Habrobracon juglandis* (Ashmead) suggested an investigation of those pigments. The only previous work on Habrobracon eye pigments was a brief chemical study, presented in abstract (PAYNE 1939), giving results but no methods or experimental data. PAYNE reported that there are at least three different eye pigments with a yellow color: a carotenoid, a flavone, and a third which yields uric acid on hydrolysis. The wild type Habrobracon eye contains all three plus melanin. She stated that the eye of the white (wh) mutant has no pigment and that ivory (o^i) has a reduced flavone which may become yellow when OH groups are added. The yellow pigment of the body is different from any of the yellows in the eye.

MATERIALS AND METHODS

The Habrobracon stocks used were wild type, white (*wh*), ivory (o^i), cantaloup (*c*), dark carrot (wh^d), carrot (wh^c), orange (*o*), and dark red (rd^d). The wild type eye is a very dark reddish-brown, almost black, color. The white and ivory eyes seem to be devoid of pigment and have a greenish tinge due to some substance beneath the eye. The cantaloup eye has an orange-pink color, carrot has a light yellow-orange color, and dark red and dark carrot both have a clear maroon red color which is not as dark as wild type.

[1] I want to thank DRS. P. W. and A. R. WHITING for supplying me with the Habrobracon used.

The wasps were etherized and decapitated about four days after hatching and the long antennae, which are black and contain a lot of pigment, were dissected off. The heads were then placed in the solvents recommended by EPHRUSSI and HEROLD: 30 percent ethanol acidified with HCl to pH2 (AEA) and absolute methanol acidified with 1 percent HCl by volume (AMA). The same quantity of eye substance, 30 heads in four cubic centimeters of solvent,

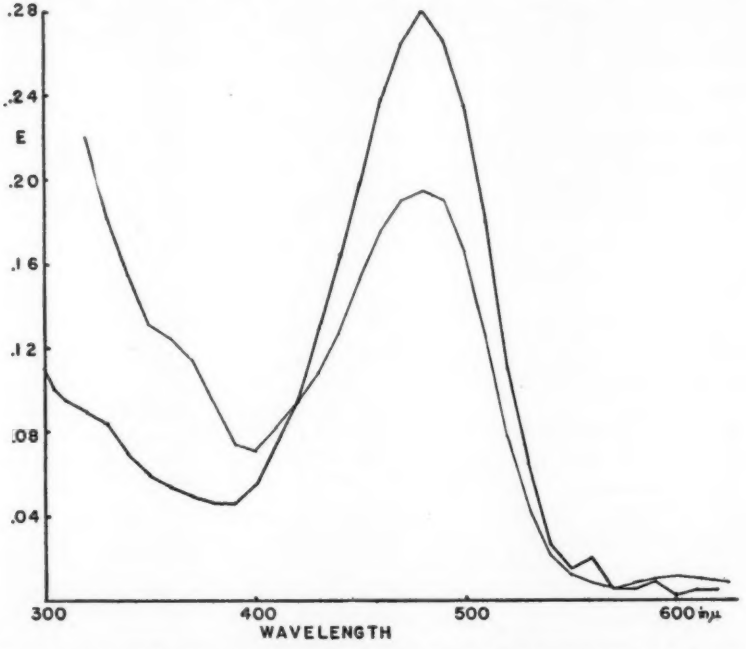

FIGURE 1.—Absorption spectra of the red pigment of Drosophila extracted with AEA. Extraction times: heavy line, 48 hrs.; light line, 24 hrs. E, the extinction coefficient, $= \log I_0/I$.

was used in each extraction. The extractions were continued 12 to 24 hours in the dark at room temperature (about 25°C). The acid ethyl alcohol solvent is stable and the same solution was used for all extractions. The acid methyl alcohol is not stable and was made fresh each time. The extract solutions obtained were perfectly clear and free of turbidity. The light absorption of the various solutions was measured with a Beckman spectrophotometer in 10 mμ intervals from 300 to 625 mμ using a 1 centimeter absorption cell.

RESULTS

The extraction procedures were first tested by extracting the pigments from *Drosophila melanogaster* wild type eyes, measuring the absorption, and

comparing the absorption curves with those obtained by EPHRUSSI and HEROLD (1944). Fifty wild type Drosophila heads were dissected off and extracted with five cubic centimeters AEA for 24 or 48 hours. The extinction coefficient E (=log I_0/I) was plotted against the wavelength (fig. 1). The curves show the same maximum at 480 mμ found by EPHRUSSI plus an increased absorption in the near ultraviolet which EPHRUSSI did not test for with his method. By testing the absorption at 485, 482, 478, and 475 mμ it was found that the point of maximum absorption of the red pigment was at exactly 480 mμ. Another 50 wild type Drosophila heads were dissected off and

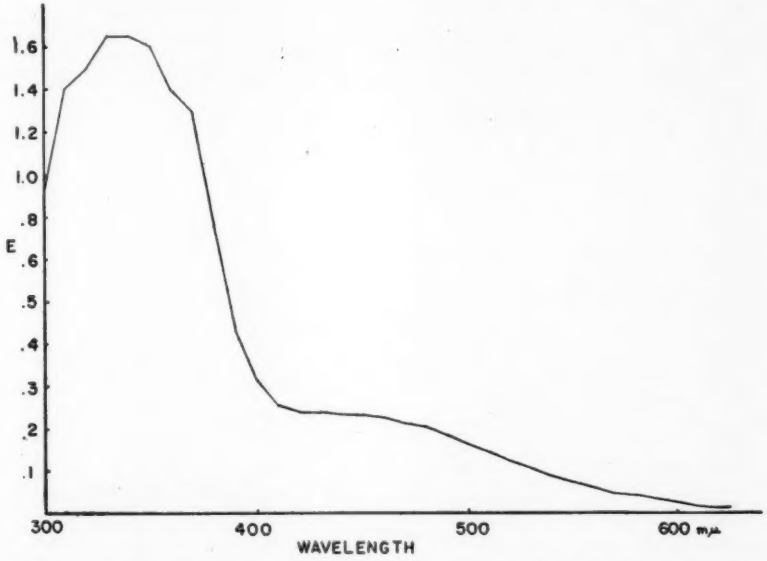

FIGURE 2.—Absorption spectrum of the brown pigment of Drosophila extracted with AMA.

extracted with five cubic centimeters AMA for 24 hours in the dark at room temperature. Figure 2 shows the absorption curve obtained by plotting the extinction coefficients against wavelength. This curve shows no sharp maximum but a plateau from 420 to 460 mμ in the visible and a peak at 335 mμ in the near ultraviolet. EPHRUSSI found a similar plateau with a slight peak at 450 mμ and said that there probably was a second peak in the near ultraviolet. WALD and ALLEN (1946) made an extract in distilled water of the brown pigment and obtained an absorption curve with a broad plateau from about 460 to 420 mμ.

The Habrobracon eye pigment is insoluble or soluble only to a very slight extent in AEA. The extracts obtained were clear and colorless and spectrophotometric analyses of the extracts from several different stocks (fig. 3) revealed

an extremely low extinction through the visible spectrum from 400 to 600 mμ and a gradually increasing absorption in the ultraviolet from 400 to 300 mμ. The absorption found is probably not due to the eye pigment but to some other substance or substances extracted from the heads, since extracts from white-eyed flies gave an absorption as great or greater than any of the extracts from flies with pigmented eyes. There was no suggestion of an increased ab-

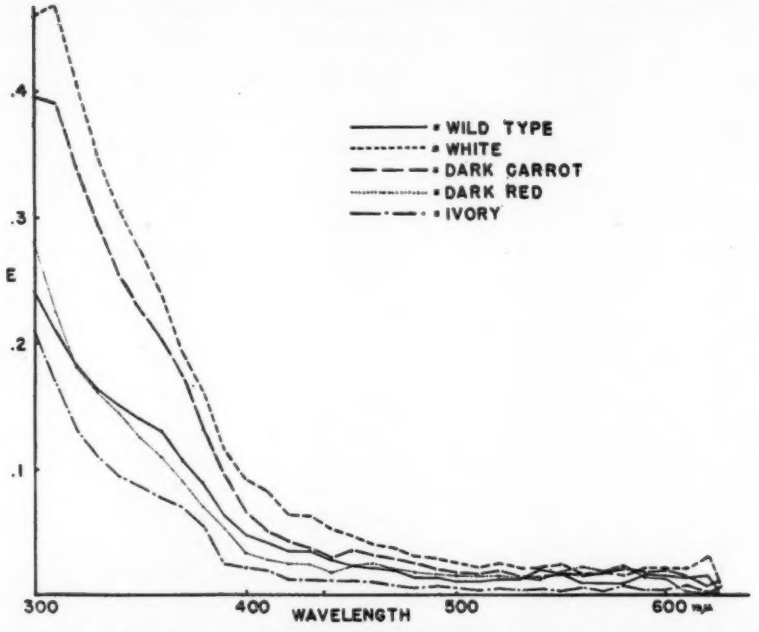

FIGURE 3.—Absorption spectra of AEA extracts of Habrobracon wild type and mutant eyes.

sorption at 480 mμ in any of the extracts so there seems to be no pigment in the Habrobracon eye which corresponds with the red eye pigment of Drosophila.

The Habrobracon eye pigments are soluble in AMA. The extracts obtained from the various stocks varied from a pale yellow to a golden brown. Spectrophotometric analysis showed that the absorption curve from the extract of wild type Habrobracon (fig. 4) is very similar to that of the brown pigment of Drosophila (fig. 2), with no sharp maximum but a broad plateau from 420 to 470 mμ. The extracts from the eyes of the several mutant stocks all gave absorption curves similar to that of wild type but with lower absorptions in the visible, indicating that these mutants, dark carrot, dark red, carrot, orange, and cantaloup, have eye pigments qualitatively similar to wild type but present in smaller amounts. Since white eyes have no pigment, the absorption curve

is due to other substances extracted from the heads. The extracts from the other eye mutants also contain these "impurities." One can get a better idea, therefore, of the absorption due to the eye pigments themselves by subtracting the extinction coefficient E for white from the extinction coefficient for wild type at each wavelength and plotting these differences against wavelength (fig. 5). These curves show essentially the same thing as figure 4: the absorp-

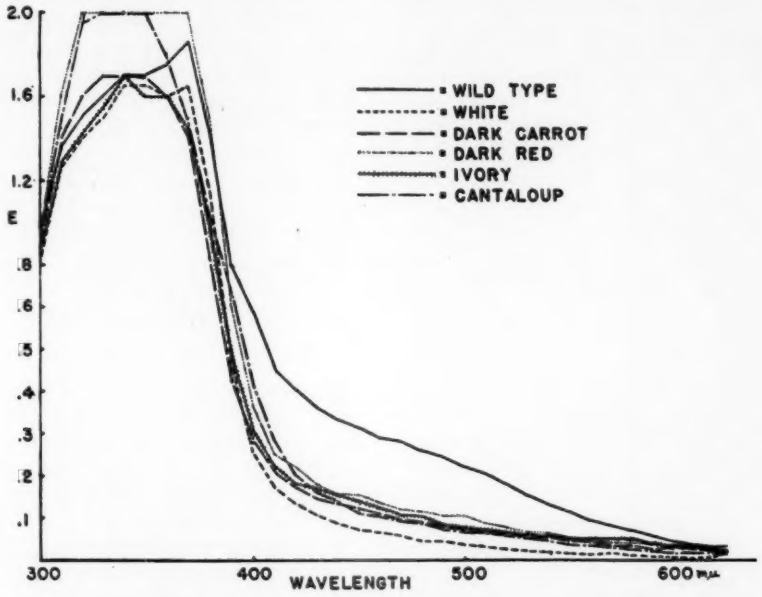

FIGURE 4.—Absorption spectra of AMA extracts of Habrobracon wild type and mutant eyes.

tion is high from 400 to 470 mμ and falls off gradually with increasing wavelength.

Figure 6 shows the absorption curves of wild type Habrobracon pigment before and after the addition of two drops of 2 percent H_2O_2. Oxidation by peroxide increases the absorption slightly from 410 to 470 mμ and decreases it slightly from 490 to 625 mμ. Treating the extracts with $Na_2S_2O_4$ causes no appreciable change in the absorption curves (fig. 7).

The Habrobracon pigment resembles the brown pigment of Drosophila in all chemical properties tested: it is insoluble in water but soluble in acid absolute methanol; it is decolorized and apparently destroyed by alkali since treatment with acid does not restore the color; its color in AMA extract changes from brown to yellow on addition of peroxide and from brown to pink on addition of sodium hydrosulfite.

DISCUSSION

These experiments show that the eye pigment of wild type Habrobracon has no component corresponding to the red pigment of Drosophila but does have one very similar to the Drosophila brown pigment. The Habrobracon eye pigment thus resembles that of the blowfly, Calliphora, in having only a

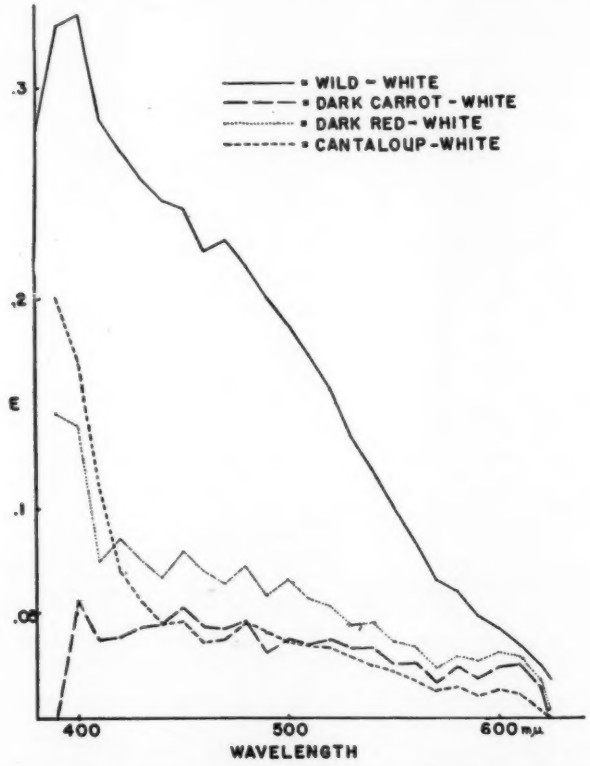

FIGURE 5.—Absorption curves obtained by subtracting the extinction coefficient for white from that of wild type or a mutant at each wavelength and plotting these differences ($E_w - E_{wh}$) against wavelength.

brown pigment and differs from Ephestia which has both red and brown pigments. Earlier evidence that the eye of Habrobracon contains a pigment similar to Drosophila brown was provided by BEADLE, ANDERSON, and MAXWELL (1938). By feeding or injecting Habrobracon extracts to Drosophila larvae, they demonstrated that wild type wasps contain both v^+ and cn^+ substances, known to be precursors of the Drosophila brown pigment, whereas orange and ivory wasps contain only v^+ substance. Reciprocal tests, the injection of

Drosophila extracts into Habrobracon larvae or pupae showed that wild type but not cinnabar Drosophila contain a substance capable of modifying ivory eye color toward wild type. They concluded that the cn^+ and o^+ genes are homologous (or "homophenic," see VILLEE, 1944) and that *cn* and *o* represent parallel mutations. Apparently no mutation in Habrobracon has occurred as

FIGURE 6.—Absorption spectra of wild type Habrobracon before (solid circles) and after (hollow circles) treatment with peroxide.

yet which is homophenic with Drosophila vermilion, since all the mutants tested by BEADLE, ANDERSON, and MAXWELL, red, cantaloup, and white in addition to the ones mentioned previously, gave positive tests for v^+ substance. Since the present study indicates that Habrobracon has no red pigment a mutation in Habrobracon homophenic with Drosophila vermilion would probably give an unpigmented, greenish-white eye resembling white and ivory.

The absorption curves of the extracts of mutant eye pigments, carrot (wh^e)

and orange (*o*) as well as the ones given in fig. 4, are all very similar to the wild type pigment but differ in the amount of pigment per eye, since all of the mutant eyes give lower absorption curves than wild type, and the amount of absorption roughly parallels the intensity of the color of the eye. None of the

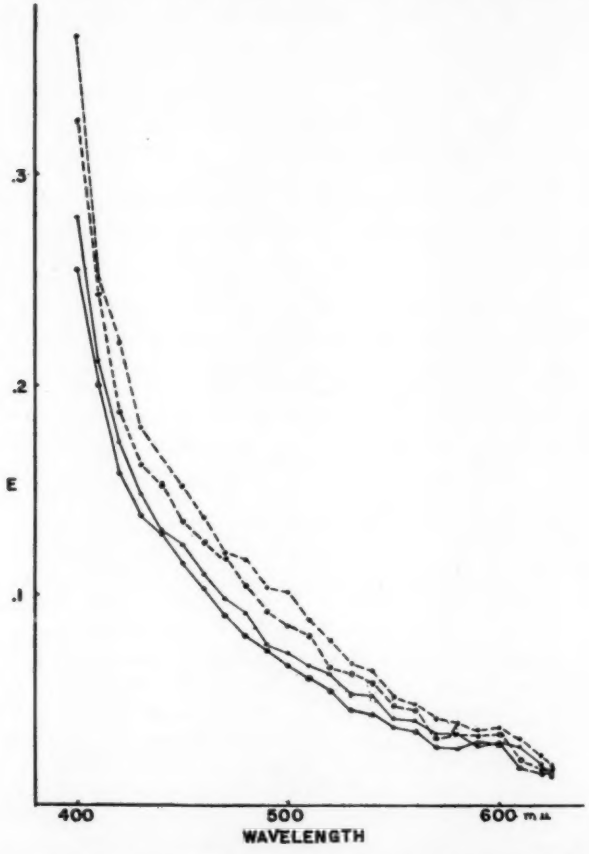

FIGURE 7.—Absorption spectra of dark red (dotted lines) and dark carrot (solid lines), extracted in AMA, before (solid circles) and after (hollow circles) treatment with sodium hydrosulfite.

mutants gave an absorption curve which differs markedly from the wild type curve as the sepia brown pigment of Drosophila differs from wild type brown (WALD and ALLEN 1946).

Nothing can be concluded from the present study about the chemical nature of the Habrobracon pigment. It would be helpful to have the chemical

data on which PAYNE (1939) based her conclusions. The fact that the absorption curve has a broad plateau rather than a sharp maximum suggests that the pigment is composed of several substances as PAYNE concluded. The red pigment of Drosophila which has a sharp maximum in its absorption curve was shown by WALD and ALLEN (1946) to consist of five different substances separable by chromatographic fractionation. Attempts have been made to chromatograph the Habrobracon pigment but without success.

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GENETIC INFLUENCES UPON THE SEX RATIO IN THE RABBIT[1]

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INTRODUCTION

ACCORDING to the generally accepted chromosomal theory of sex determination, the primary sex ratio at fertilization should be equality. The secondary ratio at birth and the subsequent tertiary ratio, however, are rarely equal. The suggested reasons for this have been summarized and discussed by PARKES (1926), CREW (1927), CRAFT (1938), and LAWRENCE (1941), to which the reader may be referred for an extended bibliography. In general they are attributed in some manner either to selective fertilization or to selective mortality of the embryos resulting either from environmental or genetic influences, most of which are very difficult to isolate specifically.

Study of the tremendous mass of data which has been recorded on sex ratios impresses one with the relatively great amount of variation, contradictions, and even confusion, which surrounds the problem and which has apparently led several students to suggest that the factors influencing the sex ratio of species and races within the species are probably numerous. Presumably it is the large number of factors affecting the sex ratio which make it so difficult in most cases to recognize specific factors; although in Drosophila, which lends itself so well to genetic analysis, clear monofactorial cases have been analysed. One cannot but wonder if perhaps adequate analysis of other species might not serve to clarify some of the irregularities. Although numerous studies have been made of the sex ratio of higher forms in hybridization, there is a striking parallel of these studies to those of the early plant hybridizers in that relatively few hybridization experiments have been carried adequately beyond the F_1 generation to demonstrate the real genetic nature of the influences involved.

The data on the rabbit described in the present communication are unique in that because they are accumulated from populations being studied for other characteristics (which studies required several generations beyond the F_1), a more complete analysis of the effects of hybridization on the sex ratio is possible than in any previous study, and this permits a little better insight into the fundamental processes involved.

MATERIALS AND METHODS

The data used in this study were obtained from 17,058 rabbits produced during the years 1937–1944 in the colony maintained for genetic studies at

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Brown University. They were obtained from seven pure races, from F_1 hybrid generations obtained by crossing them, from several subsequent F_2 and backcross generations, and from one testcross of particular significance. Approximately 6,484 of these animals were obtained from more or less heterogeneous matings, not involving the pure races, which it is believed, may be regarded as a random control population. During the period involved, several of the races achieved a considerable degree of inbreeding, in one case as much as sixteen generations (parent offspring).

All animals were sexed at birth by the method described by SAWIN *et al.* (1938) and most of them were rechecked by autopsy or by subsequent examination at one month of age. In those cases where any doubt existed as to the correct sex, the animal was not included.

TABLE 1

Sex ratio of total population according to origin.

GROUP	POPULATION	TOTAL	%♂♂	♂♂ PER 100 ♀♀	χ^2
1	Pure Races	4161	48.5	94.4	3.52
2	F_1	1117	51.2	104.6	.56
3	F_2	931	49.9	99.8	.66
4	Bx	2456	48.6	94.6	1.88
5	2–4 Bx	1550	49.1	96.5	.51
6	Bx (special)	359	46.8	88.0	1.56
7	Misc.	6484	50.2	100.9	.14
8	Totals	17,058	49.5	97.8	8.83

Total chi-square =8.83, df=7, P=.25 (approx.)
Pooled chi-square =2.02, df=1, P=.15 (approx.)
Heterogeneity chi-square =6.81, df=6, P=.35 (approx.)

For comparison with various authors the ratios obtained are expressed in both percent of males and males per 100 females.

OBSERVATIONS

The sex ratio for the entire population of 17,058 animals is $49.5 \pm .26$ percent males or 97.8 males per 100 females which is much lower than the 104.6 recorded for the rabbit by CREW (1927) or the 50.1 percent males more recently recorded by KAVEN (1937 from NACHTSHEIM).

As shown in table 1 the total population has first been separated into seven distinct subgroups. Group 1, table 1, is made up of 4,161 rabbits born to matings within seven relatively homozygous and unrelated races of rabbits. It has a ratio of 48.5 percent males which is lower than that of the population as a whole. Group 2, table 1, is composed of 1,117 animals which are first generation hybrids between members of the several races in group 1. The

percent of males (51.2) is the highest of the seven groups and is actually slightly greater than the proportion reported by CREW. Statistically the difference between groups 1 and 2 has a probability of about seven percent. It corresponds to the difference noted in species hybrids by CRAFT (1938) except for the fact that it represents an increase rather than a decrease of males. The F_2 (group 3, table 1) shows an almost exact equality of the two sexes, and the backcross (group 4, table 1) approaches very closely the value obtained for the pure races. The last two groups are more heterogeneous. Group 5 includes animals

TABLE 2

Variation in the sex ratios of the pure races.

GROUP	RACE	TOTAL	%♂♂	♂♂ PER 100 ♀♀	χ^2
1	A	347	43.2	76.1	6.36
2	X	1311	49.6	97.9	.13
3	IV	28	32.1	47.3	3.58
4	V	1538	49.9	99.5	.01
5	IIc	275	50.1	103.7	.09
6	III	624	46.0	85.2	2.00
7	XV	38	47.4	90.0	.11
8	Total	4161	48.5	94.3	12.28

Total chi-square = 12.28, df = 7, P = .09 (approx.)
Pooled chi-square = 3.52, df = 1, P = .07 (approx.)
Heterogeneity chi-square = 8.76, df = 6, P = .19 (approx.)

derived from second, third, and fourth, etc., backcrosses to the pure races and thus their genetic constitution and sex ratio are more like that of the pure races. Group 7 whose ratio approaches equality is a highly heterogeneous group comprising all other matings in the colony and because of this may be regarded more or less as a control group. Its sex ratio compares quite favorably with the ratio obtained by KAVEN, which is very close to the expected equality.

Considered as a whole the variations observed in the sex ratio in these populations could be entirely at random in a homogeneous population since neither the total nor the heterogeneity chi-squares are significant (SNEDECOR 1946).

Since the most significant change is the increase of males occurring in the F_1 generations (group 2, table 1), which tends to subside in the F_2 (group 3) and backcross generations (groups 4 and 5) to figures resembling more closely those of the parental races, it could be considered as a manifestation of hybrid vigor.

The Pure Races

Although not statistically significant, the departure from expected equality in the pure races of table 1 is sufficient to warrant further investigation. On table 2 the data for each of these races is shown separately. Although the departure from equality of the group as a whole could be due to random sam-

pling (χ^2 test of heterogeneity = 8.76, P = .19), it is seen that the greatest irregularities occur in races A, III, and IV. Those of races A and IV are statistically significant and thus suggest real genetic differences.

Hybrid Generations

Further evidence of this is to be seen in table 3 which shows the results of various of the possible crosses which were made between the seven races shown in table 2. Collectively the populations are much more uniform than were the pure races from which they arose (heterogeneity chi-square = 8.35,

TABLE 3

Variation in the sex ratios of the F_1 populations.

MATING	CROSS	TOTAL	%♂♂	♂♂ PER 100 ♀♀	χ^2	P
1	A×X*	110	52.7	111.5	.33*	>.50
2	A×V	27	40.7	68.8	.93	>.30
3	A×II$_C$	59	54.2	118.5	.42	>.50
4	A×III	21	47.6	90.9	.05	>.80
5	III×II$_C$	34	47.1	88.9	.12	>.70
6	III×IV	28	32.1	47.4	3.57	>.05
7	III×V*	267	50.6	102.3	.03*	>.80
8	III×X*	298	51.3	105.5	.22*	>.50
9	II$_C$×IV	28	42.9	75.0	.57	>.30
10	II$_C$×X	93	53.8	116.3	.53	>.30
11	V×X*	152	55.9	126.9	2.13*	>.10
12	Total	1117	51.1	104.6	.56	>.30
13	Total*	837	52.1	108.8	.15	>.70

Total chi-square = 8.9, df = 11, P = .60 *2.71, df = 4, P = .60
Fooled chi-square = .56, df = 1, P = .48 * .15, df = 1, P = .70
Heterogeneity chi-square = 8.35, df = 10, P = .60 *2.56, df = 3, P = .45

P = .60). Since in seven of these the number of individuals observed is small, it seemed desirable to consider separately the races from which the greatest number of individuals were obtained. Those have been starred, and as shown in table 3 their heterogeneity chi-square is slightly less than .45. In view of the borderline significance of these variations, it is particularly interesting to examine the influence of the several races which have contributed most to the population. Race X, which itself has almost 50 percent males, appears to maintain a high proportion of males no matter with what race it is mated. Race III by contrast has a relatively low proportion of males and in crosses with other races appears to depress the ratio. Compare, for example, mating 7, table 3 (III×V cross) with mating 11 (V×X cross) and with mating 2 (A×V). Although the numbers are not large, race IV also seems to have a depressing influence upon the sex ratio (see matings 6 and 9).

The F_2

As shown in table 4 two features of the F_2 are outstanding. First, is the fact that the F_2 populations are less variable than is the case in the F_1 (chi square test of heterogeneity is 1.19, P = .98); and second, is the reduction in

TABLE 4

Variation in the sex ratios of the F_2 populations.

MATING	CROSS	TOTAL	%♂♂	♂♂ PER 100 ♀♀	χ^2	P
1	A×III	32	53.1	113.3	.13	>.50
2	III×X	315	48.6	94.4	.26	>.50
3	III×V	252	49.2	96.9	.06	.80
4	III×IV	104	51.9	108.0	.15	>.50
5	V×X	43	55.8	126.3	.58	>.30
6	II_C×IV	110	50.0	100.0	0	1.00
7	II_C×X	75	50.7	102.7	.01	>.90
8	Total	931	49.9	99.8	.001	.98

Total chi-square = 1.20, df = 7, P = .99
Pooled chi-square = .001, df = 1, P = .98
Heterogeneity chi-square = 1.19, df = 6, P = .98

number of males corresponding more nearly to the proportion found in the parental races.

Since the parental races all tend to have low ratios, the tendency for the F_2 to return to the parental ratio undoubtedly accounts for the general decrease in variability, which is contrary to results usually found in F_2 populations which differ with respect to several quantitative genes.

TABLE 5

Variations in the sex ratios of the backcross populations.

MATING	CROSS	TOTAL	%♂♂	♂♂ PER 100 ♀♀	χ^2	P
1	III×IV	466	48.1	92.6	.70	>.30
2	III×V^V	858	50.7	102.8	.17	>.50
3	III×V^{III}	367	44.4	79.9	4.58	>.02
4	III×X	309	47.6	90.7	.73	>.30
5	II_C×X	193	48.7	95.0	.13	>.50
6	V×X	263	49.8	99.2	.004	.95
7	Total	2456	48.6	94.6	1.88	>.10

Total chi-square = 6.3, df = 6, P = .40 (approx.)
Pooled chi-square = 1.9, df = 1, P = .18 (approx.)
Heterogeneity chi-square = 4.4, df = 5, P = .50 (approx.)

Backcross Populations

When the backcross populations are considered (see table 5), again the amount of variation within the group as a whole does not appear to be significant, the chi-square test of heterogeneity being 4.42, P=.50. However, the data available in this case afford an excellent opportunity to test for the existence of specific racial differences and also to test for the possible effect of a hybrid female parent upon the sex ratio as compared with that of a hybrid male parent.

As to the racial difference, attention has already been called to the fact that races III and A tend to produce a relatively low proportion of males and that in crosses they tend to depress the sex ratio more than others. Examina-

TABLE 6

Evidence that race III possesses some genetic factor which decreases the proportion of males.

MATING		♀♀	♂♂	TOTAL	PERCENT ♂♂
F_1 ♀♀ Bx^V	III×V	423	435		
F_1 ♀♀ Bx^X	III×X	64	72		
Total		487	507	994	51.0
F_1 ♀♀ Bx^{III}	III×V	198	161		
F_1 ♀♀ Bx^{III}	III×X	98	75		
Total		783	743	532	44.4

$\chi^2=6.11$, df=2, P=.04

tion of tables 2, 3, 4, and 5 show that in general where matings have been made of one race with another and also with race III, it is the mating with race III which has the lower sex ratio. In table 6 are shown the backcross progeny of two crosses, involving race III with races V and X separated according to those produced by backcrossing F_1's to race III and F_1's (in many cases the same individual females) to the other parent, V or X, of the particular cross. The difference between the two sex ratios is the most highly significant ratio (statistically) observed in this study ($\chi^2=6.11$, P=.04). This seems to be good evidence that race III possesses some genetic factor (or factors) which depresses the sex ratio.

We have less genetic information about the depressing influence on the sex ratio of race A. In the cross of races A×X, the F_1 (mating 1, table 3) possesses an excess of males as is the case in the III×V, and III×X crosses (matings 7 and 8). In the cross of races A×III the F_1 as well as both parent races are significantly deficient. From this it might be assumed that both races are deficient for the same reason. However, the excess of males in the F_2 (mating 1, table 4) suggests that the explanation is not quite as simple as that.

Furthermore, whereas race III seems to manifest no noticeable decrease in

mean litter size (WING 1945), as compared with the other races and we have up to this time no other indication of unusual prenatal mortality, a definite tendency for some mothers of race A to have small litters has been noted, and this is apparently the reason for the fact that the race over the period of this investigation was found by WING to possess the smallest litter size though this difference was not statistically significant. Examination of the gravid uteri of eleven mothers who had previously produced several litters in which the total males were deficient has shown a total of 51 dead (in some cases degenerated) among a total of eighty foetuses which were observed at 18–25 day stages.

TABLE 7

The influence of hybrid females vs. hybrid males on the sex ratio.

MATING	♀♀	♂♂	TOTAL	PERCENT ♂♂
Backcross Using F_1 Females	1178	1118	2296	48.7
Backcross Using F_1 Males	88	88	176	50.0

$\chi^2 = .1078$, df = 2, P = .95

This is a relatively high mortality to be the result of a single sex-linked gene and probably indicates that the gene effect is either autosomal, but with a greater influence on males than females, or that it is a maternal character similar to, if not the same as, the foetal atrophy described by HAMMOND (1934).

The question of whether the hybrid male or the hybrid female has a greater influence on the sex ratio may be of some importance as an indication of where the mortality in the male sex is occurring. If the irregularity in the sex ratio is due to embryonic mortality, which parent is the hybrid perhaps would make little difference. If it is due to sperm mortality, whether the female tract is a hybrid or not might be very important. As shown in table 7, 2,296 offspring have been obtained in four of these backcross populations in which the female was the hybrid parent, and 176 have been obtained from similar matings in which the male was the hybrid. Although the difference in sex ratio of 1.39 percent obtained for the two sets of data is not statistically significant ($\chi^2 = .11$, $P = .95$), the fact that the sex ratio is lower in progeny from the hybrid mother seems to be biologically important as will be further brought out in another connection below.

The evidence that three major sorts of influences affect the sex ratio in this colony of rabbits is most clearly portrayed graphically in nine groups of histograms in figure 1. The first group at the top shows the specific racial differences in the sex ratio. The races are arranged in order of increasing mean adult body weight. Each of the other groups shows the effects upon the differences in the

sex ratio found in the parent race as they are modified in the F_1, F_2 and the backcrosses of a particular cross as indicated.

The effect of the genetic constitution responsible for the racial differences (low proportion of males) manifested in races A, III, and IV, as compared with races V, X, and II_c (see histograms of group 1, figure 1), is particularly shown by comparison of groups 3 and 4 (crosses III×V and III×X) with groups 6 and 7 (II_c×X and V and X, figure 1). Although the influence of hybridization which tends to increase the number of males is strongly manifested in groups 3 and 4, it is obviously tempered by the weak male influence of race III; and thus the number of males in the F_1 is not as great as in the IIc×X or V×X. This influence is still more strongly manifested in the backcrosses to race III. Unfortunately at the present time the F_2 and backcross generations involving the A race are not completed, but in view of the previously described evidence of a single major gene they may eventually throw interesting further light on the specific differences of these races. It is particularly interesting to note that in group 8 (the cross of races A and III), both races of which manifest the deficiency in males, the F_1 although obviously showing the effect of hybridization also shows a marked deficiency of males, and that the F_2 shows a relatively high excess of males. Although the data recognizedly are not adequate, they nevertheless suggest that the influences determining the low sex ratio in races III and A may not be genetically the same; and that by recombination recessive genes are being brought together in the F_2 which increases the vitality of males and thus their proportion.

A second obvious influence appears to be that of hybridization which is manifested in all the crosses except two (groups 2 and 5, crosses III×IV, and II_c×IV) by a higher proportion of males in the F_1 than is found in either parent race, and by a tendency for the proportion of males to return to or approach that of the parent races in the F_2 and the backcrosses. In the F_1 of the II_c×IV cross, the numbers of progeny of race IV are relatively small and the proportion of males in the F_1 is greater than the average of the two races. In the III×IV cross the number of F_1 is also small.

The third genetic influence affecting the sex ratio is that associated with the relative homozygosity or hybridity of the female parent. In table 7 attention was called to the lower proportion of males in the progeny of hybrid mothers than of hybrid fathers. Although the difference there was not statistically significant, further evidence in this connection may now be pointed out in figure 1. Here it is seen that in groups 3 and 4 involving race III (with a low male ratio) with races V and X (with normal male ratios) the backcrosses to race III have male deficiencies even greater than that of race III. Similar deficiencies are also to be noted in groups 6 and 7, both parent races of which possess a normal proportion of males. This deficiency in males in the backcross is not apparent in group 2 where both parent races are male deficient. Adequate control matings are not available since the backcrosses involving F_1 males were obtained only in the Bx^{III}III×IV and in the Bx^{x}V×X (approximately 80 young in each). However, the populations in these backcrosses derived by using F_1 females compared with those using F_1 males, show almost exactly

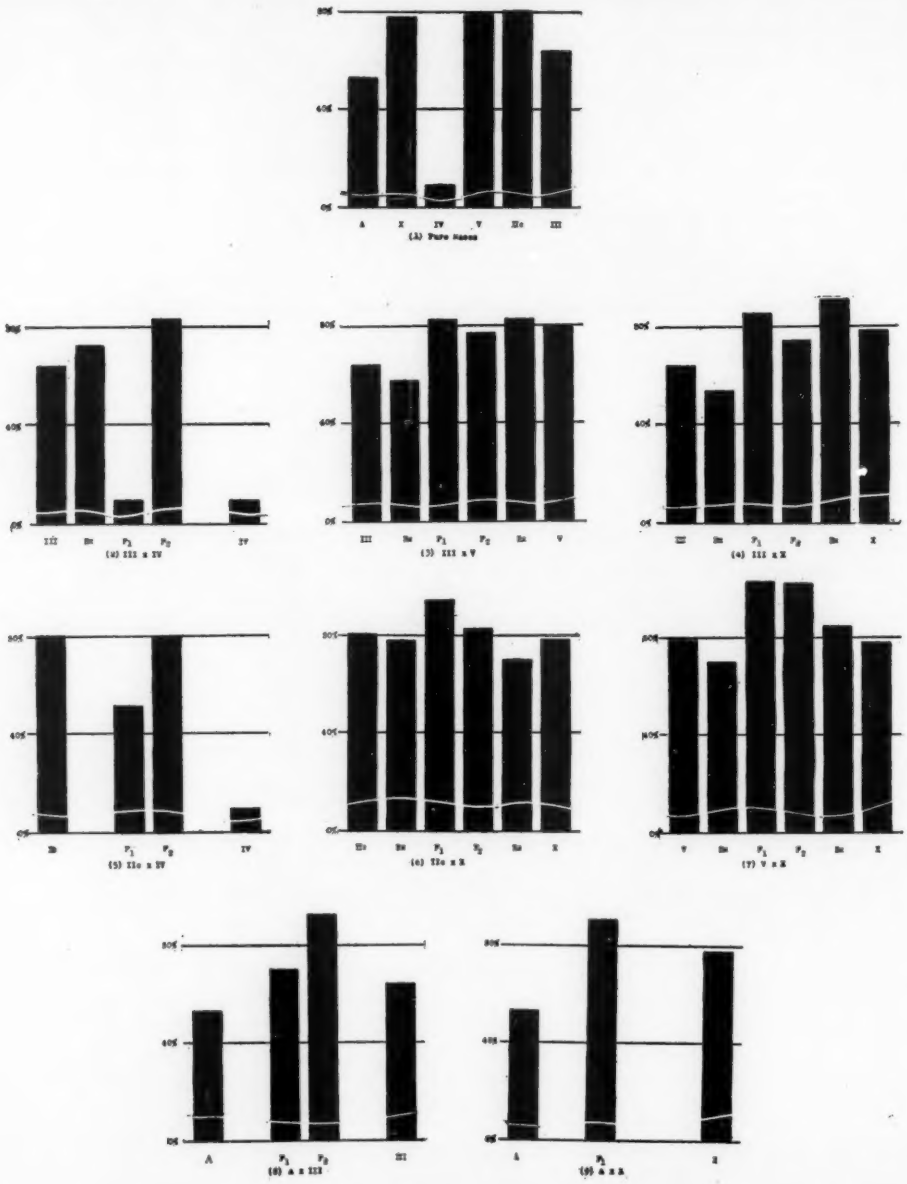

FIG. 1. See opposite page for legend.

the same difference as that found in the larger population of table 7; and thus the greater deficiencies of males noted in the backcrosses of groups 3, 4, 6, and 7 as compared with the pure races III, V, or II_c could be attributed to the fact that in those crosses the populations were derived from F_1 females. This was due principally to the fact that they were more readily available. The higher proportion of males in the $Bx^zV \times X$ is explained by the fact that the cross was made using both F_1 males and females. That of the $Bx^zIII \times X$ and of $Bx^vIII \times V$ can only be explained on the basis of the relative racial influence

TABLE 8

Sex ratio of progeny of the mating of (F_1 V×X)×III in comparison with races III, V, and X, and with their F_1 hybrids.

MATING	PROGENY	% MALES	χ^2	P
III	624	46.0	2.00	>.10
V	1538	49.9	.01	>.90
X	1311	49.6	.13	>.50
III×V	267	50.6	.03	>.80
III×X	298	51.3	.22	>.50
V×X	152	55.9	2.13	>.10
F_1 V×X×III	359	46.8	1.56	>.20

of the individual races V and X which is apparently sufficient to overbalance the sex difference resulting from the use of hybrid females.

Still further evidence of this influence of hybrid females is to be found in table 8 which shows the sex ratio of a special hybrid mating, that involving $F_1V \times X$ females with race III males, in comparison with the pure races and the usual F_1 hybrid matings of these races. This mating was made for technical reasons quite unrelated to this study of the sex ratio. It happens to be rather illuminating in this connection. Since the numbers of males in most of the previously described F_1 populations are relatively high, and this is true in the $F_1III \times V$ and III×X and particularly so of the V×X, and since the progeny resulting from mating the $F_1V \times X \times III$ should at least be genetically the equivalent of the two F_1 populations III×V and III×X, it would naturally be expected that this mating would also have a high sex ratio. Actually however, it is 46.8, closely resembling that of race III (46.0). It is unfortunate that the data are all from the one type of mating and that the critical reciprocal mating is not available as a control mating so that this point could be clearly established. However, this observation in addition to the two previous bits of evidence is highly suggestive that the relative heterozygous or homo-

FIG. 1. Percent of males in each of six pure races of rabbits (group 1, at the top) and their F_1, F_2 and backcross hybrid generations (groups 2–9). The latter are arranged with the pure races on the outside of the group, the F_1 and F_2 in the center, and each backcross next to the respective pure race.

zygous nature of the mother does have a significant influence upon the proportion of males.

In view of the case of foetal atrophy in the rabbit described by HAMMOND (1934) in which the gene action is not lethal in the usual sense (acting in the foetus) but is maternal due to the mother being homozygous for a single recessive character, it seems reasonable to suggest that a hybrid genetic milieu might also act differentially upon the threshold of viability of the two sexes.

TABLE 9

Showing the annual sex distribution in the control population.

YEAR	TOTAL	%♂♂	♂♂ PER 100 ♀♀	χ^2	P
1937	1038	50.2	100.8	.02	>.80
1938	948	49.8	99.2	.02	>.80
1939	674	51.3	105.5	.48	>.30
1940	712	50.1	100.6	.01	>.90
1941	575	49.2	96.9	.14	>.70
1942	807	51.6	106.4	.77	>.30
1943	986	48.4	93.7	1.04	>.20
1944	744	51.8	107.2	.91	>.30
Total	6484	50.2	100.9	.14	.72

Total chi-square = 3.38, df = 8, P = .91 (approx.)
Pooled chi-square = .14, df = 1, P = .72 (approx.)
Heterogeneity chi-square = 3.24, df = 7, P = .86 (approx.)

MONTHLY AND ANNUAL INFLUENCES AND PARITY

Study has also been made of the monthly and annual variation in the sex ratio among the general population which includes 6,484 individuals. No significant differences have been observed (see tables 9 and 10). With respect to parity, because of the wide variety of matings, it is difficult to find a sufficient body of data which would not be influenced in some measure by one or more of the factors noted above. However, the 890 progeny of 31 mothers, all of which had produced at least five consecutive litters by males of their own breed and representing three of the pure races, have been selected as a test of this factor (see table 11). Individually only one of the three races (race V) shows a consistent trend but collectively there appears to be a tendency for first litters to be very close to expected equality and for succeeding litters to show a gradual decline in the number of males as has been observed by numerous observers with other species. Thus, it would seem that in the younger mother, and in the older mother the male progeny are most abundant and that in middle life at the age of greatest vitality the number of males decreases.

DISCUSSION

The sex of an individual is usually regarded as being determined precisely and mechanically at fertilization; yet both the degree of sexuality within the

individual and the proportion of the sexes within the race are subject to considerable fluctuation resulting from both genetic and environmental causes. The differentiation of sex within the individual has been studied extensively

TABLE 10

Showing the monthly sex distribution in the control population.

MONTH	TOTAL	%♂♂	♂♂ PER 100 ♀♀	χ^2	P
1	593	50.1	100.3	.002	>.90
2	631	51.4	105.5	.46	>.30
3	712	49.2	96.7	.20	>.50
4	650	50.8	103.1	.15	>.50
5	553	51.2	104.8	.30	>.50
6	555	52.1	108.7	.96	>.20
7	574	50.0	100.0	0	1.00
8	418	51.4	105.9	.34	>.50
9	417	49.4	97.6	.06	.80
10	398	51.5	106.2	.36	>.50
11	493	49.9	99.6	.002	>.90
12	490	45.9	84.9	3.26	>.05
Total	6484	50.2	100.9	.14	.69

Total chi-square =6.11, df=12, P=.91 (approx.)
Pooled chi-square = .14, df= 1, P=.69 (approx.)
Heterogeneity chi-square=5.97, df=11, P=.87 (approx.)

TABLE 11

Influence of parity upon the sex ratio.

RACE	NUMBER OF MOTHERS	TOTAL YOUNG	PERCENT MALES IN SUCCESSIVE LITTERS						%♂♂ TOTAL
			1	2	3	4	5	6	
III	7	252	42.9	34.0	50.9	38.5	41.5	55.1	43.7
X	11	281	51.8	55.9	40.3	52.7	46.9		49.5
V	13	357	54.6	54.9	53.8	46.0	42.4		51.0
Total	31	890	50.6	50.3	49.1	46.5	43.6	55.1	48.4

and discussed by numerous authors but relatively little is known about specific causal factors determining irregularities in the expected proportion of the sexes in different species and races.

Two sorts of evidence from selection experiments and from hybridization studies indicate that at least a large part of such variation is hereditary. But presumably because of the number involved, specific genes are difficult to isolate. In general it is supposed that both the genetic and environmental influences causing these variations operate by way of either (1) selective fertilization or by (2) differential prenatal mortality. Evidence for the former

is found in the experiments of COLE and DAVIS (1914) and ROBERTS (1940), although in the latter case the same sort of experiment has produced no significant variation in the sex ratio in the hands of MCPHEE and EATON (1942), COLE, WALETZKY and SHACKELFORD (1940), and QUISENBERRY and CHANDIRAMANI (1940). By far the greater number of factors seem to effect the sexes differentially during prenatal development. Because the lowered vitality of the males possessing certain mutations in Drosophila has been found to be due to deleterious recessive sex-linked genes, and because of the influence which the work in Drosophila has had upon genetics in general, it has been assumed and even positively asserted that the inequalities in the sex ratio of man (LENZ 1923; HUXLEY 1924; SHIRMER 1929) and many other species (GEISER 1924–25) are induced in the same way either morphologically by structural irregularities which are incompatible with continued life, or physiologically by elevation or depression of the general threshold of metabolism and vitality of one or the other sex.

In the present study the variations in the sex ratio are not large in most cases, but in two of the pure races (III and A) departures from equality are sufficiently large to suggest the action of some one major lethal gene, either sex-linked or more effective in females. In race III there seems to be no evidence of a large prenatal mortality such as might be indicated by small-sized litters or by observations of intra-uterine mortality. In the A race, however, both of these conditions have been consistently observed in a substantial number of the mothers. Since there seems to be a very definite distinction between mothers that have and those which have not manifested these characteristics, it may tentatively be regarded as evidence of segregation of alleles of some one major gene. Whether it is a sex-linked lethal or autosomal sex-limited (having a greater influence on males than females) remains to be determined. If it should prove to be sex-linked, it would constitute the first case of a sex-linked gene of any kind in the rabbit and consequently it is being given special study at the present time.

MACARTHUR and BAILLIE (1932) object to the sex-linked lethal theory as a general explanation of variations in the sex ratio on the grounds (1) that all the most satisfactory data upon the sex ratio in Lepidoptera and Aves, in which the males are known to be homozygous for sex and thus would be expected to be longer lived, are also actually deficient in viability (see LANDAUER and LANDAUER 1931); and (2) that even in the Drosophila type forms the magnitude of the differences in mortality of the sexes does not agree quantitatively with the theoretically expected frequency of sex-linked genes based on the size of the sex chromosomes. As a rule, the males of all groups of animals except perhaps in some inter-specific crosses, appear to show the same low viability. The same evidence is inconsistent with the chromosome imbalance theory of HALDANE (1922).

SHIRMER (1929) suggested that in connection with differential mortality of the sexes, the differences can generally be best explained by assuming "that sex-linked genes affect the whole constitutional reaction"; and the studies of

GONZALEZ (1923) on Drosophila provide strong evidence that mortality is controlled through the whole genetic and environmental complex.

There seems to be little reason to believe that the same is not true with respect to prenatal mortality which is responsible for differences in the secondary ratio. GUYER (1909) thought that metabolism might be the determining factor in producing the higher sex ratio in hybrids. RIDDLE (1931) has shown that the sexes are characterized by different types and rates of metabolism regardless of the type of sex determining mechanism and this has led to a belief in a metabolic theory of the sex ratio which although somewhat vague does have some evidence to support it and does seem to explain a number of miscellaneous types of phenomena which otherwise are difficult to understand. According to it the sex difference in mortality, to use the words of MACARTHUR and BAILLIE, is considered as a natural constitutional peculiarity due to sex alone. As HOLMES (1926) has suggested the low viability of the male "is just an incidental effect of his maleness." The lethal mechanism according to this theory would operate indirectly and physiologically rather than directly and mechanically. The differences in the death rate of the sexes appear to be much more definitely associated with sex itself than with any particular nuclear equipment of sex chromosomes. Insofar as the sex ratio is concerned, the metabolic theory appears to fit particularly well with the observations of several investigators of environmental factors particularly those reported by KING and STOTSENBURG (1915) and KING (1921), by SLONAKER and CARD (1923), by PARKES (1924), by PARKES and DRUMMOND (1925), and by HOELZEL, DACOSTA, and CARLSON (1939). It also fits well with the observed results of hybridization.

SUMMER, MCDANIEL, and HEUSTIS (1922) suggested that the high hybrid ratio in deer-mouse hybrids might be due to a selection of maleproducing sperm by ova or that greater vigor resulting from crossbreeding may have influenced the metabolic rate, and HUXLEY (1924) explains the higher percentage of males of wide racial crosses as due to heterosis enabling the males to resist the deleterious effect of harmful sex limited factors. Examination of the available data on hybridization experiments shows, however, that the ratio of males is not always the highest in the F_1 but may be, as in the present study (see the III $\times$IV, III$\times$X, and III$\times$A crosses, figure 1), higher in the F_2 or in one of the backcrosses. Such an irregularity is also found in the data of GREEN (1930). He records the usual decrease in number of males in the species cross of *Mus bactrianus*$\times$*Mus musculus* but also records a relatively huge excess in the backcrosses of the F_1 to the parent species. If heterosis were always to have the effect of increasing the proportion of males in a given race, inbreeding might well be expected to reduce the number. Such a situation, however, does not always hold since MOENKHAUS (1911), SUMMER, MCDANIEL and HUESTIS (1922) in their extensive studies of Peromyscus found no significant difference between inbred and outbred lines, although in the present study it is apparent that in general the inbred races tend to have a lower ratio than do the hybrid generations as a whole. Although GREEN attached no particular significance to

it, his data also showed that in every mating involving *musculus* females the ratio is higher than that involving *bactrianus* females. These observations of differences in maternal influence are of some significance in view of the controversy which has developed over the theory of FELIX UNTERBERGER that an alkaline medium within the female reproductive tract favors the survival of males whereas an acid medium is unfavorable. This theory, although it has been tested by a number of investigators and has only been substantiated in one case (ROBERTS, 1940), is further offered by KARDIMON (1942) as an explanation of the high proportion of males reported by SEYMOUR and KOERNER (1941) in artificial insemination. KARDIMON'S explanation is further based upon the fact that natural differences in alkalinity of the cervix uteri are known to occur under varying stimuli. In view of the fact that at least three other investigators have failed to verify UNTERBERGER'S theory in laboratory animals, the value of KARDIMON'S explanation also seems somewhat questionable. However, the fact nevertheless remains that altered sex ratios have been observed in a number of cases in which there is evidence of some sort of an alteration of the biochemical condition of the mother which is suspected of being inimical to the sperm. Therefore, it seems equally reasonable to suspect that in some way the increased vitality of hybrid mothers may be exerting some biochemical influence upon the sperm. Whether the increase in vitality of the hybrid mother results in a higher threshold of the female sex hormones which perhaps are relatively inimical to maleproducing sperm or male embryos, or whether it is some other biochemical difference associated with hybrid metabolism, is an interesting speculation.

One thing that does seem clear from this study is that, as has been suspected by GONZALES in connection with mortality generally, the sex ratio is influenced by numerous factors if not by the entire genetic milieu. It also appears that the influences of hybridization can act in two ways: either directly by way of the relative viability of the male or female producing sperm or progeny; or indirectly through a maternal influence in some way altering the uterine environment as to be more suitable for the development of one sex than the other.

The irregularities with which these two are manifested in the pure races and in the hybrid populations, however, indicate that their expression is determined by way of the threshold of physiological processes which could be any one of a number of recognized influences such as hormones, alkalinity, or even nutritive differences. It seems not unreasonable to suggest that the successes or failures of attempts to alter the sex ratio which have been reported may not necessarily have been due to the alterations in acidity or alkalinity of the female tract *per se* but to some other physiological change arising from the methods of technique employed. This suggestion seems particularly pertinent in view of the observations of GUNN (1936), who found that "the longevity of spermatozoa was particularly increased by injections of the anterior pituitary-like hormone of human pregnancy urine, acetylcholine and pilocarpine."

Although it is doubtful if such methods will ever afford complete control of the sex ratio, it would seem that further study of the effects upon the sex ratio of physiological and biochemical variations of the female tract might be profitable.

SUMMARY AND CONCLUSION

Observations of the sex distribution in a population of 17,058 rabbits obtained from seven pure races, from F_1 hybrid generations obtained by crossing them, and from several F_2 and backcross generations, afford a unique opportunity to study the genetic influences responsible for departures from the normally expected equality of males and females in a laboratory mammal. Evidence of at least three major influences affecting the sex ratio is found in these animals. The most obvious appears to be that arising from hybridization or heterosis, which in six out of eight crosses increases the proportion of males beyond that found in either parental race. Of secondary importance are influences peculiar to the several specific races each of which manifests its own peculiar pattern. In one of these the irregularity is so marked as to indicate the action of either a major autosomal gene having a greater suppressive influence on the male or possibly a sex-linked gene.

A third influence is that exerted by the hybrid mother, evidence for which is found in three different types of mating, reciprocal backcross matings using F_1 hybrids mated to the parental stocks, and two kinds of special matings. In all of these cases genetic action by way of the threshold of viability of sperm or of the developing embryo is indicated and the possible relationships of these observations to the several theories as to alteration of the sex ratio are discussed. They lead to the suggestion that the successes or failures of attempts to alter the sex ratio which have been reported may not necessarily have been due to alterations in acidity or alkalinity of the female tract *per se* but to some other physiological change arising from the methods of technique employed.

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GENETICS OF NATURAL POPULATIONS. XV. RATE OF DIFFUSION OF A MUTANT GENE THROUGH A POPULATION OF *DROSOPHILA PSEUDOOBSCURA*

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INTRODUCTION

EXPERIMENTS on dispersion rates in *Drosophila pseudoobscura* have been described by DOBZHANSKY and WRIGHT (1943). These experiments, carried out during the summers of 1941 and 1942 on Mount San Jacinto, California, consisted in releasing suitably marked flies at a certain point on an experimental field, and then for several days recording the numbers of the flies that visited banana traps placed at various distances from the point of the release. The data so obtained permitted estimation of (a) average distances travelled by the flies on days with different temperatures, (b) absolute densities of wild *Drosophila pseudoobscura* on the field at the time of the experiment, and (c) rates of decline of the numbers of marked flies with time.

The drawback of the above experiments is that they describe the speed of dispersion of the released flies and the status of the wild population during only one season of the year. It should be noted that the rate of diffusion of the flies is greatly increased by increasing temperatures, and that in the mountain forests of California the fly populations reach maximal densities in midsummer.

The conditions prevailing during the seasons when the environment is less favorable to the flies remained unknown. To a geneticist the conditions during the latter seasons are most interesting. A new experiment was consequently performed in 1945–1946 at Mather, in the Sierra Nevada of California. This experiment has served in part to recheck the conclusions drawn from the older ones, and in part to furnish data of a new kind. The present article reports the outcome of this new experiment.

LOCATION, MATERIAL, AND METHODS

The experimental work has been done near Mather, at elevation of about 4600 feet, on the western slope of the Sierra Nevada of California. A description of this locality has been published by CLAUSEN, KECK, and HIESEY (1940). In brief, the vegetation belongs to a typical Transition Zone association (yellow pine, incense cedar, Kellogg oak, etc.). Winters are cold with much snowfall; summers mild and very dry. The flies are most abundant in late summer (August).

[1] Observational and experimental data by TH. DOBZHANSKY, mathematical analysis by SEWALL WRIGHT.

As in the older experiments, the third chromosome recessive gene orange has been made use of for marking the flies released at Mather. Orange-eyed flies are easily distinguishable from wild ones in the field. The flies released were F_1 hybrids of two orange strains, one extracted from the population of Keen Camp and the other from that of Andreas Canyon on Mount San Jacinto, California. By using the F_1 hybrids of these strains advantage was taken of the heterosis accruing from crossing two distantly related lines each of which has been somewhat inbred by being kept for several years in small mass cultures in the laboratory. The hybrids were raised in regular laboratory bottles, care being taken to avoid overpopulation. The fitness of the released flies is attested by the fact that they have reproduced in nature in competition with wild flies (see below, cf. also DOBZHANSKY and WRIGHT 1943). Their progeny, though diminished in numbers, has survived the winter of 1945–1946 and was present on the experimental field in summer 1946.

The techniques of trapping and recording the flies have been described by DOBZHANSKY and WRIGHT (1943) and need not be repeated here. To test the flies collected in nature for heterozygosis for the mutant gene orange, a method proposed by PROFESSOR W. P. SPENCER was employed. Tests of wild males were made by crossing individuals to laboratory females homozygous for orange. The crosses were made in "creamers" (small glass vessels) with a small amount of agar-containing culture medium. When small larvae appeared, pieces of "Kleenex" paper tissue soaked in a rich yeast suspension were placed in each "creamer." In testing of wild females from nature these were first placed singly in "creamers" and allowed to produce offspring. A sing'e son (or a single daughter) of each female was then crossed, in a fresh "creamer," to homozygous orange flies. The progeny of the crosses was inspected for presence or absence of orange-eyed flies. If the wild fly tested is homozygous nonorange, its offspring have wild-type eyes. If it is an orange heterozygote, about half of the flies in the test generation have orange eyes.

DROSOPHILA SPECIES IN THE MATHER POPULATION

The three commonest species of Drosophila in the midaltitudinal belt of the Sierra Nevada are *D. pseudoobscura*, *D. persimilis*, and *D. azteca*. These species are indistinguishable to the naked eye, and the first and the second of them are also indistinguishable under a binocular microscope.

Samples of wild flies from all collecting stations in the vicinity of Mather were examined under a microscope, and the male flies classified into *D. azteca* on one hand and a mixture of *D. pseudoobscura* and *D. persimilis* on the other. The females were not classified since they are not as easily distinguishable as the males are. The resulting data are shown in table 1.

D. azteca becomes more and more frequent relative to the other two species as the season progresses, starting with about 20 percent early in June and reaching about 50 percent in late August. It may be noted in this connection that *D. azteca* inhabits chiefly the Transition and the Upper Sonoran life zones of the Sierra Nevada, and that Mather is not far from the upper altitudinal limit of its range.

Two methods of discrimination were used to distinguish *D. pseudoobscura* and *D. persimilis* in our samples. Wild females were allowed to produce offspring, and the salivary glands of the resulting larvae were examined for chromosomes. Chromosomes of the two species differ in the gene arrangement in some sections (TAN 1935). This is the cytological method. Wild males, or sons of wild females, were outcrossed to orange *D. pseudoobscura* females. If the wild male belongs to the species *D. pseudoobscura* the sons are normal, while

TABLE 1

Number of flies of different species and sexes trapped in different seasons.

DATE	♀♀	*pseudoobscura* ♂ + *persimilis* ♂	*azteca* ♂	PERCENT *azteca*
July 8–15, 1945	425	350	197	36
August 10, 1945	—	102	65	39
August 22–September 5, 1945	611	562	572	50
June 4–15, 1946	1230	589	129	18
June 26–30, 1946	201	141	60	30
August 9–10, 1946	292	124	103	45

TABLE 2

Relative frequency of D. pseudoobscura and D. persimilis.

DATE	METHOD	*pseudo-obscura*	*persimilis*	PERCENT *pseudo-obscura*
July 8–15, 1945	Cytological	97	58	63
August 22–September 5, 1945	Genetical	267	170	61
September 5, 1945	Cytological	57	49	54
June 4–15, 1946	Genetical	1275	666	66
June 4–15, 1946	Cytological	89	35	73
August 9–10, 1946	Cytological	79	33	71
Total		1864	1011	64.8

sons of *D. persimilis* males are sterile interspecific hybrids. The sterile hybrids can easily be distinguished from normal males under a high dry power of a compound microscope in unstained squash preparations of freshly dissected testes. This is the genetic method. Table 2 reports the results.

Roughly 65 percent of the total population of *D. pseudoobscura* and *D. persimilis* belong to the former species. The proportions in the different samples are not quite uniform ($\chi^2 = 13.45$, probability of chance occurrence for five degrees of freedom about 0.02), but there is no pronounced seasonal change. The figure 65 percent may, then, be taken as characteristic for the locality.

CONTROL EXPERIMENT

Although homozygous orange-eyed flies have never been found in natural populations, the recessive gene orange is the commonest among striking visible mutant genes carried in concealed condition in both *D. pseudoobscura* and *D. persimilis*. Strains of both species descended from single females collected in nature have repeatedly proved to be heterozygous for orange. Unfortunately, no complete record of these occurrences has been kept. It can be stated, however, that orange heterozygotes occur in different parts of the geographic distribution areas of both species.

Since our main experiment consisted in liberating orange-eyed flies in the Mather locality and in studying the dispersal of the orange homo- and heterozygotes, it was evidently necessary to know how frequent orange heterozygotes were in this locality before the start of the experiment. Samples of wild flies were accordingly collected on the experimental field-to-be between July 8 and 15, 1945. A part of these wild flies, 385 in all, w .s tested by crossing them to homozygous orange flies. In 384 of these tests the offspring consisted of wild type flies, and in one test both wild type and orange-eyed flies appeared. Since each fly carried two third chromosomes, a total of 770 wild third chromosomes were thus tested, and one of them was found to carry orange.

At the time when these control crosses were being made it was not known that *D. persimilis* as well as *D. pseudoobscura*, two morphologically indistinguishable species, occur together in the Mather population. It is, consequently, known neither how many individuals of each species there were among the 385 specimens tested for orange, nor to which species the single orange heterozygote belonged. It has been found later that approximately 65 percent of the obscura-like flies found in the Mather locality are *D. pseudoobscura* and 35 percent are *D. persimilis* (see table 2). The most probable estimate is, then, that among the 770 tested third chromosomes about 500 belonged to *D. pseudoobscura* and 270 to *D. persimilis*.

In the summer of 1946, more than 750 individuals of *D. persimilis* were tested by outcrossing to orange *D. pseudoobscura* flies. None of them proved to be orange heterozygotes. This shows that the *D. pers milis* population at Mather contains few or no orange mutants. Assuming, then, that the orange heterozygote found in 1945 was a *D. pseudoobscura*, it is probable that 1 out of 493 *D. pseudoobscura* third chromosomes, or about 0.2 percent, contained the orange mutant gene before the start of the experiment. This value, 0.2 percent, will be taken as the "control value" for the frequency of orange in the Mather population of *D. pseudoobscura*.

RELEASE OF ORANGE-EYED FLIES

Between 6.25 and 6.50 P.M. on July 16, 1945, a total of 3840 orange-eyed *D. pseudoobscura* flies were liberated in a grove of old oak trees (*Quercus Kelloggii*) near Mather. On six following evenings (July 17–22), banana traps were exposed and the numbers of orange and nonorange flies visiting them were recorded. The traps were arranged in a single file, north and south from the point of the release, at distances of 20 meters from each other.

The recorded numbers of the orange and nonorange flies found in each trap on each of the six days are shown in table 3. Each entry in this table consists of two figures separated by a dash. The first figure indicates the number of orange and the second that of wild (nonorange) flies. Thus, the entry "33–21" for trap No. 3 on the third day of collecting (July 19th) means that 33 orange and 21 nonorange flies visited this trap on that day. The wild flies here recorded are, of course, a mixture of the three species, *D. pseudoobscura*, *D. persimilis*, and *D. azteca*.

Trap No. 0 was placed at the point of release, at the center of the experimental field. Traps Nos. 1–30 stood north and traps Nos. 31–60 south of the center (see table 3.) Therefore, the distances from trap No. 0 to No. 30, and from No. 0 to No. 60, were 600 meters each, and from No. 30 to No. 60 a total of 1200 meters.

DISTRIBUTION OF WILD FLIES

Inspection of table 3 shows that the wild flies were distributed sufficiently uniformly over the experimental field so that at least a single fly was recorded in each of the 61 trap locations on at least one day. Much greater numbers were, however, caught in some traps than in others. On considering the days separately, wild flies were absent from only 35 of the 351 trap records. Data on the total numbers of flies caught on successive days, and on average numbers found per trap, are given in table 4. These data are compared in table 5 with the analogous data from the four experiments (numbered I to IV) made on Mount San Jacinto and described by DOBZHANSKY and WRIGHT (1943).

It appears from table 4 that the mean number of wild flies caught per trap at Mather rose almost threefold during the six days, a change which might be due either to actual increase in the density of the population, to increased activity, or merely to more favorable temperature at the time of trapping on the later days. The standard deviation of the numbers per trap showed a closely similar increase. If the variations were due merely to accidents of sampling the distribution of numbers per trap should be of the Poisson type with the variance equal to the mean. As shown in the last column, the variance was much greater than can be accounted for as accidents of sampling although, as shown in table 5, the ratio $\overline{\sigma^2/m}$ was less than in any of the experiments on Mount San Jacinto.

That the local heterogeneity, indicated by high $\overline{\sigma^2/m}$ was due to conditions that had some degree of persistence is shown by the correlation between numbers caught on different days in the same trap. These correlations are given in table 6 according to the interval and are compared with averages from the San Jacinto data. The grand average for the 15 correlations in the Mather data is +0.518, very similar to the average of +0.545 based on 19 correlations from San Jacinto. The average correlation at intervals of one or two days is in both cases somewhat greater than at longer intervals indicating that the heterogeneity was not due entirely to persistent local conditions.

It can be concluded that wild flies occur in all parts of the experimental field on which traps were exposed, but that some neighborhoods are relatively more

TABLE 3

Numbers of orange and wild flies in different traps.

TRAP NO.	1 DAY	2 DAYS	3 DAYS	4 DAYS	5 DAYS	6 DAYS
30	—	—	0–1	0–0	0–0	1–2
29	—	—	1–0	0–0	1–0	2–5
28	—	—	0–1	0–5	0–13	0–10
27	—	—	0–2	1–2	0–1	1–3
26	—	—	2–5	1–8	1–4	0–3
25	0–0	0–1	0–0	0–0	0–0	0–0
24	0–2	1–2	0–1	0–1	3–2	1–3
23	0–8	0–1	0–10	2–8	1–6	1–7
22	0–1	0–0	0–7	0–2	1–3	1–8
21	0–2	0–2	0–1	1–9	0–1	0–12
20	1–0	0–1	0–2	0–2	0–3	1–1
19	0–0	0–0	0–0	0–1	1–0	4–5
18	0–3	0–0	0–4	0–2	0–3	1–4
17	0–4	0–5	1–9	0–5	2–2	0–3
16	1–4	0–5	1–11	2–6	3–6	0–7
15	0–1	0–5	0–5	1–2	0–2	0–7
14	0–0	2–1	1–0	1–0	1–1	1–2
13	0–2	0–2	2–1	1–5	0–1	1–4
12	0–0	0–0	1–2	1–2	2–0	1–2
11	1–0	3–2	5–2	3–0	1–5	5–7
10	1–2	3–2	2–3	2–9	3–6	5–7
9	1–0	0–4	13–4	4–5	6–5	7–5
8	0–0	7–5	6–2	2–4	2–5	6–7
7	0–2	2–2	4–6	9–5	8–8	7–7
6	3–2	16–8	18–13	12–7	34–21	19–38
5	5–3	6–2	13–9	12–10	12–12	6–12
4	5–2	8–3	26–9	21–10	26–16	25–33
3	28–11	26–12	33–21	26–20	16–17	17–38
2	23–12	39–9	46–14	23–12	18–17	12–36
1	26–2	25–3	22–11	18–2	20–10	16–13
0	129–7	78–4	92–4	36–6	47–12	36–20
31	29–4	29–5	27–5	25–5	18–5	11–6
32	33–6	44–7	53–9	37–12	13–6	57–43
33	39–9	25–5	47–9	49–16	21–14	13–16
34	7–1	19–0	22–1	25–9	11–4	11–10
35	3–3	3–2	11–1	6–7	7–2	11–7
36	6–1	6–4	14–5	8–4	3–3	6–8
37	5–0	7–1	3–1	6–3	3–2	15–16
38	2–17	2–1	6–1	6–2	4–2	3–2
39	0–1	2–5	8–8	7–12	2–5	9–7
40	0–1	0–3	2–1	0–4	0–0	6–4
41	1–2	0–8	1–3	2–3	2–5	0–6
42	1–3	2–0	3–10	2–3	2–3	3–23
43	0–1	1–1	2–1	1–0	1–1	2–11
44	0–2	1–11	1–9	0–5	2–10	2–10
45	0–4	1–4	3–3	1–7	2–3	5–28
46	0–7	1–5	1–3	1–9	0–5	2–5
47	0–3	1–16	0–11	1–9	0–5	0–15

TABLE 3—(*continued*)

TRAP NO.	1 DAY	2 DAYS	3 DAYS	4 DAYS	5 DAYS	6 DAYS
48	0–4	0–8	2–7	1–6	3–4	3–11
49	1–0	0–4	2–5	0–2	1–3	2–3
50	0–3	0–2	2–3	1–6	0–9	0–5
51	0–0	0–1	0–6	0–5	1–7	0–8
52	0–4	0–3	0–8	0–6	2–4	2–4
53	0–6	0–4	3–20	0–7	1–24	0–14
54	0–5	0–2	1–11	0–11	0–9	5–10
55	0–24	0–10	1–37	1–9	1–18	0–15
56	—	0–0	0–5	1–18	1–10	0–10
57	—	0–0	0–4	1–3	1–1	1–4
58	—	0–1	0–2	0–6	0–7	0–2
59	—	0–4	0–8	0–14	0–9	1–5
60	—	0–3	0–6	0–5	0–4	0–5
Total	351–181	360–201	504–363	361–358	311–366	347–624
t° (F)	—	70°	71°	72°	72°	72°

TABLE 4

Statistics on numbers of the wild flies caught on successive days at Mather. The temperature (F) at time of collection, number of traps set, number of wild flies caught, the mean number (m) per trap, the standard deviation ($\bar{\sigma}$) of the number per trap, and the ratio σ^2/m are shown.

DAY	TEMP.	TRAPS	WILD FLIES	m	σ	σ^2/m
1	—	51	181	3.5	4.5	5.8
2	70°	56	201	3.6	3.4	3.2
3	71°	61	363	6.0	6.2	6.4
4	72°	61	358	5.9	4.5	3.4
5	72°	61	366	6.0	5.6	5.2
6	72°	61	624	10.2	9.9	9.5

TABLE 5

Comparison of data on numbers of wild flies caught at Mather and in four experiments on Mount San Jacinto. $\bar{m}$ is the unweighted average of the daily averages of the numbers of wild flies caught per trap, $\bar{\sigma}$ is the similar average for the standard deviation of the numbers and $\overline{\sigma^2/m}$ is the similar average for the ratio, σ^2/m.

EXPERIMENT	DAYS	$\bar{m}$	$\bar{\sigma}$	$\overline{\sigma^2/m}$
Mather	6	5.9	5.7	5.6
San. Jacinto I	9	5.9	7.4	9.7
San. Jacinto II	7	29.3	17.5	10.6
San. Jacinto III	7	21.2	17.5	15.0
San. Jacinto IV	5	21.7	11.3	5.9

attractive to the flies than others. Estimates of the absolute densities of wild flies at Mather are given below (table 10).

DISTRIBUTION OF RELEASED FLIES

Table 3 shows that the released orange-eyed flies were recaptured mainly in the vicinity of the point where they were liberated. About 88 percent of the orange flies found one day after the release at Mather were found in the seven traps that were 60 meters or less from the point of release, although one orange fly was caught 400 meters to the north and another 380 meters to the south. By the sixth day, the proportion of orange flies in the central seven traps had fallen to 47 percent and one was caught 600 meters to the north (at the end of the line of traps) and one 580 meters to the south. It should be said that the region of release was one that was somewhat above the average in attractiveness for *D. pseudoobscura*. This is indicated by the fact that 24 percent of the wild flies were caught in the central seven traps during the six days, although these constituted only 12 percent of the traps set during the period.

TABLE 6

Correlations between numbers of wild flies caught in the same trap locality on different days. The results for every pair of days were calculated for the Mather data. Only the correlations between the first and subsequent days were calculated for the four experiments on Mount San Jacinto.

LOCALITY		INTERVAL									
		1 DAY		2 DAYS		3 DAYS		4 DAYS		5 DAYS	
		DAYS	r	DAYS	r	DAYS	r	DAYS	r	DAYS	r
Mather		1–2	+0.377	1–3	+0.678	1–4	+0.459	1–5	+0.482	1–6	+0.308
		2–3	+0.562	2–4	+0.490	2–5	+0.457	2–6	+0.449		
		3–4	+0.516	3–5	+0.751	3–6	+0.462				
		4–5	+0.577	4–6	+0.591						
		5–6	+0.604								
	Av.	(5)	+0.527	(4)	+0.628	(3)	+0.459	(2)	+0.466	(1)	+0.308
San Jacinto											
	Av.	(4)	+0.645	(4)	+0.616	(4)	+0.598	(3)	+0.488	(4)	+0.381
Total	Av.	(9)	+0.579	(8)	+0.622	(7)	+0.538	(5)	+0.479	(5)	+0.366

The orange flies dispersed equally to the north and south. The mean location of capture (m) was never more than about 20 meters from the point of release and was north of the latter on some days, south on others (table 7) the unweighted average of the six means was only 0.8 m. south of the point of release although the total range reached on the sixth day was 1180 m.

The dispersion of the released flies on the experimental field can be described in terms of the variance (σ^2) of the distance at which these flies are found from the point of release on successive days of the experiment (DOBZHANSKY and WRIGHT 1943). If the flies scatter over the field at random, and

equally fast on all days, variance should increase in proportion to the time elapsed since the release of the orange flies. The variance (in meters^2) observed on successive days is as follows:

1 day —4051	4 days—15444
2 days—7252	5 days—23378
3 days—14202	6 days—28377

A comparison of the standard deviations (σ) in the Mather experiments with those found in the four experiments performed on Mount San Jacinto is shown in table 7. The Mather figures are about equal to those in experiments II, III, and IV on San Jacinto performed at similar temperatures. The stand-

TABLE 7

Comparison of the Mather experiments with those on San Jacinto with respect to standard deviation in meters (σ) and kurtosis (Ku) of released flies along the lines of traps. The number (n) of orange flies recaptured on each day and the center of location in meters north (+), or south (−) of the point of release (m) are also given for the Mather data.

	MATHER				SAN JACINTO							
					I		II		III		IV	
DAY	n	m	σ	Ku	σ	Ku	σ	Ku	σ	Ku	σ	Ku
1	351	− 5.6	64	13.0	39	9.8	59	7.6	58	10.4	68	8.3
2	360	+ 1.2	85	7.7	57	5.7	92	5.0	94	4.4	95	5.9
3	504	− 4.7	119	7.9	74	4.2	102	4.4	131	2.8	136	4.2
4	361	− 3.8	124	7.8	72	4.3	117	3.6	129	3.0	177	4.0
5.	311	+20.5	153	6.1	64	4.5	122	4.0	133	2.7	171	3.0
6	347	− 8.1	169	5.4	84	3.5	159	3.6	171	1.8	—	—
7	—	—	—	—	93	3.1	161	2.7	190	1.9	—	—
8	—	—	—	—	114	2.4	—	—	—	—	—	—
9	—	—	—	—	97	3.9	—	—	—	—	—	—

ard deviations are much lower in experiment I on San Jacinto during which the temperatures were much lower than in all other experiments.

The reliability of these figures is, however, questionable, because the variance of dispersion along a line of traps passing through the point of release does not adequately indicate the amount of dispersion unless the distribution of captures is normal. Departures from normality are indicated sufficiently accurately for our purpose by the ratio of the fourth moment about the point of release to the square of the second moment, which is three in the case of the normal distribution. It was shown that this ratio was far greater than three on the first day after release in all of the San Jacinto experiments and only approached (or fell below) three several days later. The data at Mather show the same trend but much higher values on all days (table 7). High kurtosis indicates that the dispersive movements were heterogeneous: short range wandering movements on the part of most flies but relatively long flights by

some. The fourth moment is, of course, greatly affected by a few extreme distances and thus appears much less than it actually is, if the line of traps does not cover the entire range. The high kurtosis even on the sixth day at Mather may reflect the relative adequacy of the range sampled (1200 meters at Mather, 500 meters in experiment I (relatively adequate because of slow dispersion), 960 meters in experiment II, 920 meters in experiment III and 1080 meters in experiment IV. The subnormal values of the kurtosis on the sixth and seventh days in experiment III especially suggest curtailment of the range (table 7).

Whatever heterogeneity there may be with respect to dispersion, there should be the same contribution on each day to the mean square radial distance from the point of release, if direction of movement is random and if the distribution of radial distances which flies cover is the same on each day. The

TABLE 8

Estimates of variance in kilometers² (σ^2) and standard deviation in kilometers (σ) for the whole population in one direction $\sigma^2 = \frac{1}{2}\Sigma r^2 f/(\Sigma r\bar{f} + c/2\pi)$ in Mather and in San Jacinto experiments on each day. Temperature in F°.

DAY	MATHER			SAN JACINTO											
				I			II			III			IV		
	t°	σ^2	σ	t°	σ^2	σ	t°	σ^2	σ	t°	σ^2	σ	t°	σ^2	σ
1	?	0.0095	0.098	56	0.0032	0.056	70	0.0073	0.086	70	0.0085	0.092	71	0.0106	0.103
2	70	0.0126	0.112	67	0.0050	0.071	71	0.0128	0.113	72	0.0128	0.113	72	0.0152	0.123
3	71	0.0264	0.162	66	0.0074	0.086	70	0.0136	0.117	77	0.0176	0.133	74	0.0239	0.155
4	72	0.0275	0.166	59	0.0072	0.085	71	0.0160	0.127	71	0.0177	0.133	78	0.0394	0.199
5	72	0.0392	0.198	55	0.0061	0.078	68	0.0190	0.138	74	0.0173	0.132	69	0.0286	0.169
6	72	0.0433	0.208	65	0.0080	0.090	73	0.0299	0.173	75	0.0215	0.147			
7				62	0.0087	0.093	63	0.0254	0.160	74	0.0285	0.169			
8				63	0.0119	0.109									
9				60	0.0105	0.102									

mean square radial distance for a radially symmetrical frequency distribution is given theoretically by the expression $\int_0^{2\pi}\int_0^{\infty} r^3 z dr d\vartheta / \int_0^{2\pi}\int_0^{\infty} r z dr d\vartheta$ where r is the radial distance, z the corresponding ordinate of the frequency function (inadvertently omitted in the formula as given in the preceding paper) and $(rdrd\vartheta)$ the element of area. As brought out in the preceding paper, an approximation can be obtained by calculating $\sum r^3 f/(\sum rf + c/2\pi)$ where f is the mean frequency at distance r and c is frequency at the point of release.

The dispersion variance that is of most interest, however, is that of the whole population in a single direction. The north-south variance involves not only the observed dispersion along the line of traps through the point of release but the dispersion along all lines parallel to this. With kurtosis greater than three, the variance along these parallel lines is greater than that along the line through the center. This total variance in one direction should be just half the radial variance discussed in the preceding paragraph. Table 8 shows this variance (in kilometers²) and the standard deviation (in kilometers) in relation to temperatures on each day at Mather and in the four experiments

on San Jacinto. The variance in one direction increased about 0.007 km.^{2} per day at Mather and in experiment IV, at about half this rate (after the first day) in experiments II and III, and only by about 0.001 km.^{2} per day in experiment I. It appears that it would require some five months for the standard deviation in one direction to reach one kilometer under the most favorable conditions found in these experiments. On comparing the standard deviations estimated for the whole population in table 8 with those observed along the line of traps through the point of release (table 7) it may be seen that the latter (on reduction to kilometers) are usually smaller, especially on the early days when kurtosis was high. The average ratio in the eight cases in which Ku is greater than 7.5 is 0.70, in six cases to which Ku is from 4.5 to 6.1 is 0.80, in the 11 cases in which Ku is from 3.5 to 4.4. is 0.89, in the six cases in which Ku is from 2.7 to 3.1 (close to the normal value 3.0) it is 1.00, and in the three cases in which Ku is markedly subnormal (1.8 to 2.4) it is 1.11. This illustrates the point that the observed standard deviation along a line of traps through the point of release should agree with the standard deviation of the whole population in the same direction only if the distribution is a bivariate normal one.

POPULATION DENSITY

The total number of flies that would be caught in a grid in which traps are spaced at 20 m intervals in parallel lines can be estimated from the formula $K[2\pi\sum r\bar{f}+c]$, where K is a constant that is less than one if captures are reduced by the presence of parallel lines of traps (DOBZHANSKY and WRIGHT, 1943). These estimates are shown in the second column of table 9.

TABLE 9

The number of orange flies which it is estimated would be caught in a grid of traps at 20 m intervals in parallel lines 20 m apart by the formula $(2\pi\Sigma rf+c)K$, and the ratio of this estimate to the total number actually released. This ratio is also given for the four experiments at San Jacinto.

MATHER			SAN JACINTO			
			I	II	III	IV
DAY	ESTIMATE	RATIO	RATIO	RATIO	RATIO	RATIO
Released	(3840)	100	100	100	100	100
1	2230K	58K	19K	50K	61K	66K
2	3300K	86K	63K	53K	94K	63K
3	6270K	163K	50K	42K	81K	33K
4	4850K	126K	22K	58K	45K	31K
5	5010K	131K	11K	31K	71K	23K
6	6370K	166K	36K	44K	47K	—
7	—	—	15K	48K	34K	—
8	—	—	16K	—	—	—
9	—	—	5K	—	—	—

If conditions were the same on all days, these figures should fall off at the same rate as the whole population of released flies. Instead of this, the estimates for

all later days are greater than that on the first day and the maximum is reached on the last day. However, the captures of wild flies per traps show an even greater increase. This general parallelism makes it probable that conditions were more favorable for trapping or that the activity of the flies was greater on the later days. In the preceding paper the estimated numbers of orange flies capable of being captured in a system of traps such as described was divided by the ratio of wild flies per trap to that on the first day to correct for activity. The data from San Jacinto, whether corrected or not, indicated a statistically significant falling off in the orange population at a rate of about nine percent per day. The Mather data show a rise to the third day followed by a greater decline when corrected, in contrast with the three fold increase when uncorrected. They give no aid, however, in estimating the true rate of decline.

More disconcerting perhaps is the fact that on all days from the third to the sixth the estimated number of orange flies capturable in a 20 m grid came out much greater than the actual number of orange flies released, except for the competition factor K. Only one such case occurred in the experiments on San Jacinto. This may mean that K is less than 0.5 (flies being attracted from greater distances than indicated before) or else that there was more dispersion along the line of traps than at right angles to it, contrary to the assumption of a radially symmetrical dispersion. On San Jacinto dispersion was demonstrated to be more or less radially symmetrical by the use of a cross shaped arrangement of traps. This was not done at Mather but there was nothing in the terrain to suggest channelling of dispersion in one direction. It is possible however that we have overestimated somewhat the total amount of dispersion at Mather.

The density of the wild population at Mather may be estimated for comparison with those made from the San Jacinto experiments. It will be assumed as before that the released population decreased 9.2 per cent per day as estimated from the San Jacinto data. This means an estimate of 3840×0.908^n on the nth day after release. Independent estimates can be obtained for each day by the formula (DOBZHANSKY and WRIGHT 1943)

$$\text{wild}/400\text{m}^2 = \text{K}(\text{wild}/\text{trap}) \times 3840 \times 0.908^n / \text{K}(2\pi r\bar{f} + c).$$

The term $\text{K}(2\pi r\bar{f} + c)$ is the estimated number of orange flies capturable in a 20 m grid, such as discussed above. It is assumed that the wild flies actually caught per trap should also be multiplied by K to give the corresponding estimate for wild flies capturable per trap (each at the center of an area of 400 m^2) in such a grid. The K's cancel. These estimates (divided by 4 to give density in terms of flies per 100 m^2) are given in table 10 including all days on San Jacinto instead of merely the first two previously published. In averaging these for each experiment, the figures for each day have been weighted by the term $n_1 n_2/(n_1+n_2)$, where n_1 and n_2 are the total numbers of wild and orange flies captured. The estimates for Mather, July 16–July 22, 1945, average 0.9 flies/100 m^2, and are consistently lower than on San Jacinto (3.8 flies/100 m^2 in

early June 1942, 9.8 flies/100 m² in mid-June 1942, 6.7 flies/100 m² in early July 1942 and 8.9 flies per 100 m² in late July 1942).

The conclusion that the population density of *D. pseudoobscura* at Mather is lower than it is on San Jacinto is much strengthened if one recalls that the figures for "wild flies" given in table 10 for Mather refer to a mixture of *D. pseudoobscura*, *D. persimilis*, and *D. azteca*. Only *D. pseudoobscura* occurs on Mount San Jacinto. About 36 percent of the flies caught in July of 1945 at Mather were *D. azteca* (table 1), and about 35 percent of the remainder were *D. persimilis* (table 2). Hence, only about 42 percent of the "wild flies"

TABLE 10

Estimates of the density of the wild population based on the captures on each day of wild flies as compared with captures of orange flies released in known numbers and assumed to decrease at a rate of 9.2 percent per day. The averages for all days are based on weights depending jointly on the total numbers of wild (n_1) and of orange (n_2) flies caught on each day. $[wt=n_1n_2/(n_1+n_2)]$.

DAY	MATHER		SAN JACINTO							
			I		II		III		IV	
	WT.	FLIES PER 100 m²	WT.	FLIES PER 100 m²	WT.	FLIES PER 100 m²	WT.	FLIES PER 100 m²	WT.	FLIES PER 100 m²
1	119	1.39	66	3.42	294	8.34	376	8.52	531	8.22
2	126	0.91	238	4.22	237	8.53	369	6.39	214	7.51
3	199	0.74	164	3.62	186	9.85	228	5.03	88	11.65
4	163	0.78	77	3.58	221	9.47	139	7.25	65	10.97
5	157	0.73	24	1.51	123	13.26	105	5.04	47	14.15
6	216	0.95	111	3.01	70	9.93	51	4.08		
7			50	3.38	74	14.00	37	6.86		
8			50	4.32						
9			22	8.83						
Av.	980	0.89	802	3.80	1205	9.76	1305	6.67	945	8.86

caught in July 1945 belonged to the species *D. pseudoobscura*. If the population density of "wild flies" per 100 square meters was 0.89 (table 2), the figure for *D. pseudoobscura* becomes about 0.37 of a fly per 100 square meters. This is less than one-tenth of the population density in midsummer on San Jacinto (table 10). The relative rarity of the flies at Mather compared to San Jacinto was realized from the start of the experiments in the former locality because the absolute numbers of flies visiting the traps there were strikingly smaller.

MASS RELEASE OF ORANGE FLIES

On July 23, 1945, the trapping of the flies was discontinued because some of the orange flies liberated on July 16 (see table 3 and page 310) had reached, and probably gone beyond, the ends of the trap lines. Trap lines longer than 1200 meters could not be constructed with the available number of collectors.

From July 23 till August 11 inclusive, approximately 1000 orange-eyed flies per day were liberated at the same point at which the orange flies were released on July 16. A grand total of about 25,134 orange flies were thus set free. Liberation of such numbers of flies on a single evening would, of course, raise unduly the population density of the flies near the point of release. Releasing them gradually was designed to permit the environment to absorb the newcomers. The hour of the release, between six and seven PM, was adjusted to let the orange flies out when the wild flies were active in the same neighborhood.

Between August 10 and 16 inclusive, groups of ten to 15 traps were exposed in the vicinities of the points lying 250, 500, 750, and 1000 meters north and south from the point of release, 1250 and 1500 meters south, and near the point of release itself. Since several traps placed very closely together attract much fewer flies than the same number of traps spaced at distances of more than ten meters apart (DOBZHANSKY and EPPLING 1944), the traps were placed near trees or bushes in irregular files approximately perpendicular to the north-south axis of the experimental field. No collections could be made at 1250 and 1500 meters north of the point of release because of the rugged terrain there (Tuolumne Canyon). Owing to the small number of collectors, the trapping could not be made simultaneously at the different points. The 500, 750, and 1000 meter points were sampled first, then the 0 and 250 points, and finally the 1250 and 1500 meter points. Only a single collection was made at 250 and 1500 meters, while near the point of release the trapping continued for four days. This partly explains the very unequal number of flies collected at different stations (table 11). The flies that visited the traps were, as usual, liberated where collected (see DOBZHANSKY and WRIGHT 1943).

Table 11 shows that in mid-August 1945 the adult population near the point of release consisted of decidedly more orange than nonorange flies. Since only about 42 percent of the "wild flies" actually belong to the species *D. pseudoobscura* (see above), there is no doubt that orange-eyed individuals constituted more than half of all individuals of this species which visited the traps within a circle with a radius of 500 meters centered on the point of release. The proportions of orange in the total population decreased, however, as the distance from the point of release increased. The decrease of the frequency of orange was more rapid southward than northward from the center. This may seem to indicate that the flies traveled northward more frequently than they did southward, but such an inference is not necessarily correct. Indeed, the density of the population of wild flies was greater in the territory south of the point of release than it was north of the same point. Hence, if orange flies disperse uniformly in all directions from the point of release, a greater relative frequency of orange is expected to be found in the territory in which wild flies are less abundant. Although orange flies tend to show the same preferences for different microenvironments as wild flies do, their distribution seems to be somewhat more uniform (table 11).

The problem now to be considered is how the distribution of the orange flies observed between August 10 and 16 compares with that found between July

17 and 22 (see above). The best way of computing the variance from the data presented in table 11 is to use the ratios of the numbers of orange and wild flies trapped at the various collecting stations. The use of the ratios obviates in part the complications due to varying densities of the fly population and variable numbers of traps in different parts of the experimental field. These ratios are included in table 11. It is also assumed that collecting at 1250 and 1500 meters north of the point of release would have given the same numbers of orange flies as found at 1250 and 1500 meters south of this point.

On August 10–16, 1945, the variance of distribution of the orange-eyed flies along the line of traps turns out to be 0.086 kilometers^2, and the correspond-

TABLE 11

Numbers of orange and wild type flies collected between August 10 and 16, 1945, at different distances (in meters) from the point of release.

DISTANCE	DAYS OF COLLECTING	TOTAL ORANGE	TOTAL WILD	RATIO ORANGE/WILD	ORANGE PER DAY
Point of release	4	674	208	3.24	168.5
250 North	1	43	18	2.39	43.0
250 South	1	40	21	1.90	40.0
250 Total	2	83	39	2.13	41.5
500 North	7	48	42	1.14	6.86
500 South	7	46	134	0.34	6.57
500 Total	14	94	176	0.53	6.71
750 North	4	6	23	0.26	1.50
750 South	4	12	96	0.13	3.00
750 Total	8	18	119	0.15	2.25
1000 North	7	12	139	0.09	1.71
1000 South	5	6	100	0.06	1.20
1000 Total	12	18	239	0.075	1.50
1250 South	3	6	110	0.055	2.00
1500 South	1	1	103	0.010	1.00

ing standard deviation 0.293 kilometers. These figures should be compared with the variance and standard deviation observed on the sixth day of the initial experiment (July 22, cf. table 7) which are 0.028 kilometers^2 and 0.169 kilometers respectively. The variance has, consequently, trebled between July 22 and August 10–16. The kurtosis of the distribution on August 10–16, measured as before, is 7.5 or somewhat higher than that on July 22. Because of this high kurtosis, an estimate of the variance of the whole population in one direction, made with the aid of the formula $\sigma^2 = \frac{1}{2}\sum r^2\hat{f}/(\sum r\hat{f} + c/2\pi)$, comes out nearly twice as great as that along the line of traps, viz., 0.156 kilometers^2. The standard deviation of this dispersion is 0.395 kilometers.

Another way to compute the variance is to take in consideration only the collecting stations at 500 meters, 1000 meters, and at the point of release. This gives the variance 0.055 kilometers^2 (table 15), which is an underestimate because it is computed from a truncated distribution; however, it has the ad-

vantage of being comparable to the figure for the August 22–September 5 collecting (see below).

The orange-eyed individuals which came to the traps exposed on the experimental field between August 10 and 16 were doubtless recaptures of the flies liberated at the center of this field between July 16 and August 11. Very few, if any, orange-eyed progeny of the released parents could have hatched from pupae and be old enough to enter traps by mid-August. Furthermore, since the longevity of the flies in natural habitats is much lower than in the laboratory (DOBZHANSKY and WRIGHT 1943), most of the flies recaptured between August 10 and 16 must have been liberated only a few days before the recapture. The observations made in mid-July showed the variance of the distribution of orange flies to increase at a rate of approximately .0047 km^2 per day along the line of traps (see above). The higher of the two estimates of variance for mid-August seems, consequently, to be about what we might expect if the variance continued to grow at a uniform or accelerated rate. An acceleration is indeed expected because late July and the first half of August were warmer at Mather than mid-July. Since the rate of dispersal of flies increases with temperature, the flies released in August must have traveled for relatively greater distances.

SAMPLING IN LATE SUMMER OF 1945

Between August 22 and September 5, 1945, samples of the population were taken again in the neighborhood of the point of release, and at 500 and 1000

TABLE 12

Numbers of orange and wild type flies collected between August 22 and September 5, 1945, at different distances (in meters) from the point of release.

DISTANCE	DAYS OF COLLECTING	TOTAL ORANGE	TOTAL WILD	RATIO ORANGE/WILD	ORANGE PER DAY
Point of release	8	274	515	0.532	34.25
500 North	7	39	231	0.169	5.57
500 South	7	27	302	0.089	3.86
500 Total	14	66	533	0.124	4.71
1000 North	2	3	105	0.029	1.50
1000 South	9	4	113	0.035	0.44
1000 Total	11	7	218	0.032	0.64

meters north and south from it. The numbers of orange and normal-eyed flies recorded at this time are given in table 12. Comparison of tables 11 and 12 discloses that during approximately two weeks which elapsed between the two samplings the proportions of orange flies in the adult population on the experimental field have dwindled very appreciably. This is doubtless explained by death of many of the released flies. On the other hand, some of the orange flies found in late August and early September were the progeny of the releaesd parents which developed outdoors. This was established by inspecting some

of the flies under a microscope; at least two undoubtedly young flies with orange eyes were found among about one hundred inspected ones.

The variance computed from the data in table 12 is 0.092 kilometers^2, and the standard deviation 0.293 kilometers (table 15). This value for variance is only slightly higher than that obtained for the August 10–16 data, namely 0.086 kilometers^2 (see above). However, this value represents undoubtedly an underestimate because of the curtailment of the range over which collections were made (only at the point of release, at 500, and at 1000 meters in August 22–September 5, also at 250, 750, 1250, and 1500 meters on August 10–16, cf. tables 11 and 12). A fairer comparison can be obtained by calculating the variance for the earlier date from the same collecting stations. This comes out 0.055 kilometers^2, or considerably below that for August 22–September 5. A very appreciable increase of the variance during the second half of August is expected, because the warmest period of the summer was reached at about the middle of August and toward the beginning of September the weather became much cooler. Still another estimate can be obtained as follows. The estimate of the variance for the whole population on August 10–16 is 0 156 kilometers^2, or 2.8 times greater than the variance along the line of traps, 0.055 kilometers^2. Multiplying the figure for variance on August 22–September 5 (0.092) by 2.8, we obtain 0.258 kilometers^2 as the variance, and 0.51 kilometer as the standard deviation, on August 22–September 5 (table 15). This is probably an overestimate since it involves the assumption that kurtosis late in August remained as high as it was at the earlier date.

The released orange flies have interbred with the native wild ones. Copulating pairs consisting of two orange, one orange and one wild and two wild individuals were observed repeatedly in the traps in the course of the experiment. Since the presence of some young orange-eyed flies was recorded on the experimental field between August 22 and September 5, some orange heterozygotes must have been present there at that time. Accordingly, some of the phenotypically wild type flies collected on the field were shipped to the laboratory in New York and tested for heterozygosis for orange. The wild males were crossed singly to virgin laboratory-bred orange-eyed females. The wild females were allowed to produce offspring, and a single son or a single daughter of each female was outcrossed to orange. Presence or absence of orange flies in the next generation shows whether or not the wild type parent was heterozygous for orange. Each cross tests two third chromosomes present in a fly. Since both *D. pseudoobscura* and *D. persimilis* occur at Mather, a male from the progeny of each outcross to orange was dissected and its testes were examined under the microscope. The progeny of *D. persimilis* flies outcrossed to the orange mutant of *D. pseudoobscura* are sterile hybrids, and their testes are easily distinguishable from those of males of either pure species. The results obtained are summarized in table 13. Since approximately 0.2 percent of third chromosomes in flies found on the experimental field before the release of the orange flies carried the mutant gene orange (see control), the observed percentages of the orange-containing third chromosomes must be corrected by subtracting 0.2 percent.

Since very few chromosomes were tested and few heterozygotes were found, calculation of variance from the data in table 13 does not seem to be justified. All that these data show is that orange heterozygotes were present on the experimental field in late August and early September of 1945, and that they

TABLE 13

Numbers of third chromosomes of Drosophila pseudoobscura tested and of those carrying orange. Flies collected between August 22 and September 5, 1945.

DISTANCE	CHROMOSOMES TESTED	ORANGE	RATIO ORANGE/WILD	PERCENT ORANGE
Point of release	130	11	0.092	8.46
500 North	14	3	0.049	4.69
500 South	74	3	0.042	4.05
1000 South	36	1	0.029	2.78

were more common in the vicinity of the point of release than away from this point.

DISTRIBUTION OF ORANGE IN JUNE OF 1946

Flies were collected again in the vicinity of the point of release and of the points 500 meters north and south of there between June 4 and 15, 1946. On June 26 and 30 collections were made at approximately 1000 meters north and south from the point of release. No orange-eyed flies were found among several thousand individuals examined. The absence of orange homozygotes does not, however, preclude the possibility that individuals heterozygous for this gene were present.

Accordingly, wild males were crossed in individual cultures to orange females, and the progeny was examined for presence or absence of orange-eyed flies. One son of each male was dissected and its testes were inspected under a microscope to distinguish between the cultures which had *D. pseudoobscura* and those which had *D. persimilis* fathers. Wild females were allowed to produce offspring, and a son or a daughter of each female was outcrossed to orange. The progeny was also examined for orange, and a single grandson of each wild female was dissected to determine the species to which its wild ancestor belonged. The data thus obtained are summed up in table 14.

The data in table 14 disclose the very significant fact, namely that the orange-carrying chromosomes were still clustered about the point of release in June of 1946, or about ten months after the liberation of the orange flies. The observed deviations from a uniform ratio of orange to wild flies would occur by accidents of sampling with a probability of less than 0.01 ($\chi^2 = 10.3$, two degrees of freedom).

The variance along the line of traps comes out 0.182 kilometers^2, or almost exactly double the figure obtained for the August 22–September 5, 1945, sampling. Even if the variance for June 1946 be rated up by the factor 2.8 to give an estimate for the whole population, again assuming persistence of the kur-

tosis 7.5 (see above), the resulting figure for the variance is only 0.510, indicating a standard deviation of only about 0.7 kilometer about ten months after the release of the flies (table 15).

The variance observed in late August and early September of 1945 was reached as a result of dispersal of orange flies liberated from two to six weeks

TABLE 14

Numbers of third chromosomes of Drosophila pseudoobscura tested and of those carrying orange. Flies collected between June 4 and 30, 1946.

DISTANCE	CHROMOSOMES TESTED	ORANGE	RATIO ORANGE/WILD
Point of release	646	18	0.0287
500 North	746	13	0.0177
500 South	698	12	0.0175
500 Total	1444	25	0.0176
1000 North	334	2	0.0060
1000 South	312	1	0.0032
1000 Total	646	3	0.0047

TABLE 15

Variance (in kilometers^2) and standard deviation (in kilometers) along the line of traps considering only the samples taken at 0, 500, and 1000 meters from the origin. In the last two columns the variance and standard deviation are estimated for the whole population by multiplying the variance in the second column by 2.8 (see text).

MATERIAL	DATE	TRAPS		POPULATION	
		σ^2	σ	σ^2	σ
Flies	August 10–16, 1945	0.055	0.24	0.156	0.40
Flies	Aug. 22–Sept. 5, 1945	0.092	0.29	0.258	0.51
Chromosomes	June 4–30, 1946	0.182	0.43	0.510	0.72

previously. This variance was only a little more than doubled during the nine and a half months that elapsed between early September of 1945 and mid-June of 1946. The evident lack of strict proportionality between dispersal and time is not at all strange because the rate of dispersal is greatly modified by temperature. Below 50°F the flies move very little if at all (DOBZHANSKY and EPLING 1944). Since freezing temperatures occur at Mather during winter (CLAUSSEN, KECK, and HIESEY 1940), there can be little migration of flies for about five or six months each year. The migration rates during spring and autumn must be low, and only during July and August the high temperatures induce rapid dispersal.

DISCUSSION

Despite the high sampling errors involved, some conclusions are clearly justified by the data presented above. The experiments performed on Mount San Jacinto (DOBZHANSKY and WRIGHT 1943) and in Mather (described in

the present article) agree in showing that in a fairly uniform two-dimensional environment *D. pseudoobscura* flies disperse more or less at random. To be sure, some microenvironments, such as proximity of old oak or pine trees, are clearly attractive to wild as well as to laboratory-bred flies. Nevertheless, we have never found discrete foci of concentration of the flies in nature, nor have we observed directional movements resembling those described by TIMOFÉEFF-RESSOVSKY and TIMOFÉEFF-RESSOVSKY (1940) in *D. funebris* and *D. melanogaster*, and interpreted by these authors as due to the influence of wind. It is fair to note in this connection that all our experimental fields were in localities remarkably free of strong winds during the seasons when the field work was in progress.

The rate of dispersal varies greatly with temperature. As judged by the failure of the flies to come to traps at temperatures below 50°F, the movements of the flies are negligible in cold weather. On days with temperatures of about 70°F at the time of the evening activity of the flies, the average distances between the locations of a fly on successive days are close to 120 meters. Values close to 200 meters per day are probably reached with evening temperatures of about 78°F.

It is, therefore, understandable that in the Mather experiment the amount of dispersal during August was greater than during July, while from September till June the flies traveled only as much as they did during a part of July and August. DUBININ and TINIAKOV (1946) believe that *D. funebris* near Moscow has a period of rapid migrations during June (which is a relatively cool month), followed by a period of a more sedentary existence later in the summer when temperatures are as a rule higher. There is nothing in our data to indicate such alternation of migratory and sedentary phases in *D. pseudoobscura*. The published data of DUBININ and TINIAKOV do not, in our opinion, prove such an alternation in *D. funebris* either. The very interesting experiments of these authors consisted in releasing flies homozygous for a certain inversion and in observing its distribution on a territory two kilometers long and 200 meters wide. In about 60 days after the release, populations about one kilometer distant from the origin showed mixtures of inversion homozygotes and heterozygotes in proportions approaching the binomial square ratios. If released and wild flies interbreed at random, such proportions can be formed in a single generation, not in two generations as DUBININ and TINIAKOV (1946, p. 542) supposed. The results of DUBININ and TINIAKOV are compatible with the assumption that the dispersal of *D. funebris* occurs at rates resembling those found in *D. pseudoobscura*.

If the flies disperse at random, the variance of their distribution increases, with temperature held constant, in proportion to the time elapsed since the release. The standard deviation, and the average distance at which a released fly or its progeny are found from the point of origin, increase as the square root of the time interval. Thus, if flies disperse at a rate of m meters per day on the average, they will be found after n days at an average distance of $m\sqrt{n}$ meters from the origin. As a consequence, the rate of diffusion of a mutant gene through a population is fairly slow even in such relatively mobile but randomly

moving forms as *D. pseudoobscura*. Orange-eyed flies were released at Mather between July 16 and August 11, 1945. About ten months later, between June 4 and 30 of 1946, more than half of the progeny of the released flies was still concentrated within a circle with a radius of one kilometer from the origin. Although some further dispersion doubtless took place in July of 1946, there can be no doubt that within a year the flies and their progeny have not moved very far from the point of release.

To help a nonmathematical reader visualize the observed rate of diffusion of the gene orange, the following very crude figures can be mentioned. We take the figure 0.72 kilometers to represent the standard deviation (in one direction) of the distribution of the progeny of orange flies about ten months after their release (table 15). This is probably an overestimate for ten months, but may be fairly close as an estimate of the standard deviation one year after the release. Now, if the progeny of the flies one year after the release is normally distributed, then half of this progeny will be found within a circle with a radius of about 0.85 of a kilometer from the origin. About 95 percent of the progeny will be found within a circle with a radius of 1.76 kilometers, and about 99 percent of the progeny within a circle with a radius of 2.2 kilometers.

SUMMARY

3840 orange-eyed flies were liberated at a certain point near Mather, California, on June 16, 1945. On the six following days traps were exposed along a line 1200 meters long running through the point of release, and the numbers of orange and wild flies visiting these traps were recorded. Analysis of the data confirms the conclusions reached from similar experiments made earlier on Mount San Jacinto (DOBZHANSKY and WRIGHT 1943). At temperatures close to 71°F, the variance of the distribution of flies increased at a rate of about .007 square kilometers per day in one direction, reaching a standard deviation of .21 kilometers in the six days.

More orange-eyed flies were released at the same point near Mather between July 23 and August 11, 1945. A total of 25,134 flies were thus set free. Between August 10 and 16, the standard deviation of the distribution of orange flies on the experimental field was estimated to lie between 0.24 and 0.40 kilometers. Two weeks later the standard deviation rose to between 0.29 and 0.51 kilometers.

Between June 4 and 30, 1946, flies were collected at the point of release and at 500 and 1000 meters north and south of this point. No orange homozygotes were found but some flies proved to be orange heterozygotes. The concentration of orange heterozygotes was higher near the point of release than further away from this point. The standard deviation of the distribution of heterozygotes is estimated between 0.43 and 0.72 kilometers. Taking the higher estimate this means that ten months after the release of the orange-eyed flies about 95 percent of their progeny are found within a circle with a radius of 1.76 kilometers or less centered on the point of release.

The population density of wild *D. pseudoobscura* in midsummer at Mather is found to be around 0.4 of a fly per 100 square meters of the territory. This is

only about one-tenth to one-twentieth of the density found in the corresponding season at Idyllwild, on Mount San Jacinto.

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POPULATION GENETICS OF A POULTRY FLOCK UNDER ARTIFICIAL SELECTION

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THE subject of population genetics first received attention shortly after the rediscovery of MENDEL's work. In the last thirty years purely deductive studies of the consequences of Mendelian heredity in biological populations have been carried out by many investigators, culminating in the definitive summaries of FISHER (1930) and WRIGHT (1931 and 1942). The principles derived in this fashion are being gradually tested by observations and experiments under both natural and laboratory conditions. Much of this work has been summarized by HUXLEY (1940) and by DOBZHANSKY (1941) with particular reference to the evolutionary aspects of population genetics.

The same principles have been applied to problems of breeding and improvement of domestic animals by WRIGHT (1939) and more particularly by LUSH (1945) and his students. The development of systematic breeding plans designed to control and manipulate the forces acting in population dynamics have proceeded largely on the basis of the deductive studies mentioned above, with but little direct aid from experiments on either laboratory or domestic animals.

Not only are properly designed experiments with animals of economic value expensive to conduct, but the relatively long span between generations places a definite limitation on the study of long-range changes in the genetic makeup of such populations. Most herds and flocks have not been maintained for sufficiently long periods of time, and records generally are not complete enough for a satisfactory study. Suitable material for a study of some of these problems is found in the complete pedigree and production records which have been kept on the production-bred Single Comb White Leghorn flock at the UNIVERSITY OF CALIFORNIA from 1933 to date. Using these records of this flock we compared some of the actual changes which have occurred in the flock with those expected to occur on the basis of current theories of population genetics.

The forces causing genetic changes in biological populations have been identified as selection, migration, chance and mutation. The material at hand permits a thorough study of the first of these. Some information is available on the rôles of migration and of chance. The effects of mutation could not be investigated directly, but these must have been small in view of the low mutation rate commonly observed in laboratory experiments and of the relatively small number of generations. Hence, the specific objectives of the present

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study were to examine the changes which have occurred in our material in relation to the rôles of selection, migration and chance.

FLOCK HISTORY AND THE BREEDING PLAN

The flock originated in 1933 from birds kept for various experimental purposes. At that time and for several years previously the average production in the first laying year per original pullet housed at five months of age fluctuated around an average of 120 eggs, roughly similar to that observed then on commercial egg-production farms in California. The actual averages were 117.5 eggs in 1931, 118.6 eggs in 1932 and 125.6 eggs in 1933, when part of the flock was set aside for selection for high egg production. Thereafter, a systematic plan of family- and progeny-testing in breeding for productive characters was pursued under the direction of DR. LEWIS W. TAYLOR and the senior author.

Several males originating from other flocks were introduced during the course of the years; however, only the introductions made in 1933 and 1934 were incorporated into the flock to any large extent. Females from other groups of birds on the University plant were used as breeders in the production flock occasionally until 1941, since which date the flock has been entirely closed.

The number of pullets each year varied from 402 to 704, except in 1944 when only 260 were retained because about half the flock was diverted to another experiment. The birds were hatched in March and April each year, care being exercised to maintain their pedigree identity throughout life. Pullets were housed at about five months of age and no culling was practiced until the end of the first laying year.

The essential features of the breeding plan were:

1) emphasis in selection was largely placed on sister and progeny performance;
2) with a few exceptions in the early stages the breeding was from hens two or more years old;
3) as already noted, occasional outcrosses were made in the early years of flock formation, the flock being closed after 1941;
4) the size of the flock varied from 400 to 700 pullets each year from approximately 11 sires and 85 dams, with the previously noted exception in 1944;
5) mating of closely related individuals was consciously avoided.

Breeding plans similar to this appear to be fairly common in the field. The senior author has had occasion to examine the pedigree records of a number of California flocks. Several resembled rather closely those of the flock under study. An additional feature that this flock has in common with commercially bred flocks is the fact that the number of eggs laid was not the sole criterion of selection. Such characters as sexual maturity, egg size, egg quality, hatchability, and viability were also considered. The over-all effect of including these characters was to reduce the selection intensity for egg production. Thus the improvement obtained in egg number is not the maximum but rather that which is feasible under commercial breeding conditions where the number of eggs laid is not the exclusive criterion of worth.

IMPROVEMENT IN EGG PRODUCTION

The average annual hen-housed egg production of the flock from 1933 to 1944 is shown in figure 1. The decrease during the period from 1937 to 1939 which gives the appearance of a cyclic trend was caused by increased mortality from lymphomatosis (TAYLOR, LERNER, DEOME and BEACH 1943), presumably due to greater exposure to the disease. This factor and a consequent reduction in selection for egg production due to additional emphasis on resistance to lymphomatosis may account for the considerable drop in the production index in 1938. The slope of a linear regression fitted to the data indicated an annual change in production of 5.6 eggs per year. Although the trend is not

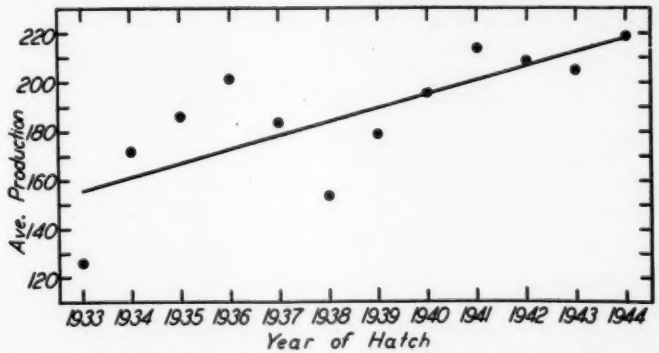

FIGURE 1.—Average production index by years in the UNIVERSITY OF CALIFORNIA production-bred flock. The flock had an increase in incidence of lymphomatosis in 1937–38. The slope of the least squares' regression line is 5.6 eggs per year.

necessarily uniform throughout the twelve years, this figure may be accepted as the average increase in egg number per year.

It is possible to compare the observed rate of improvement with the rate expected on the basis of *a priori* information in this population. The agreement between the actual and the expected rates would partially test whether or not the genetic principles of breeding for egg production are correctly understood. The expected rate of improvement depends on the 1) intensity of selection; 2) accuracy of selection, i.e., heritability; and 3) interval between generations which is measurable in terms of the age of parents (DICKERSON and HAZEL 1944). Estimates of all three of these factors were available in this population, and from them the expected rate of improvement was computed.

Selection intensity can be expressed in terms of the selection differential (the average of the selected birds minus the average of the whole group in which they were hatched). Selection was based chiefly on three criteria: individual performance, records of sisters, and records of progeny. The first two were applied to hens when first selected for mating (since nearly all dams had completed a year of laying before being mated), and the third to hens which were

remated after their progeny had been tested. Sires were first used for breeding when one year old, their selection being based primarily on the incomplete records of their sisters, while older sires were selected on basis of their progeny tests.

TABLE 1

Selection differentials, heritabilities and expected genetic gains from the three stages of selection.

STATISTIC	BASIS FOR SELECTION	SIRES	DAMS
Selection differential	Individual performance	—	99.1
	Sisters' record	26.0	39.8
	First-year progeny record	24.4	29.0
Heritability	Individual performance	—	0.045
	Sisters' record	0.155	0.138
	First-year progeny record	0.452	0.155
Expected genetic gain	Individual performance	—	4.46
	Sisters' record	4.03	5.49
	First-year progeny record	11.03	4.50

Table 1 presents in its first part the average selection differentials for four years (1935, 1937, 1939 and 1941) used as a sample representative of the whole period. It may be seen that selection differentials based on sisters' records were greater for dams than for sires. Although the number of dams was nearly eight times as great as that of sires, the completed records of sisters were available for them as against incomplete records for the sisters of sires mated for the first time. The selection differentials for the breeding birds selected on the basis of progeny records were also higher for the dams than for sires (29.0 vs. 24.4 eggs). This presumably is due to the fact that a smaller number of offspring was obtained from dams than from sires. In consequence of this smaller number, the variation in progeny averages was much wider for dams than for sires and the same percentage of culling on progeny-test information gave a larger selection differential (in actual units) for the dams than for the sires.

The advantages shown in the records of selected birds were in part due to differences in environment, exposure to disease, or other intangible but non-hereditary factors. The regression expected to occur must be considered in computing the genetic gain accomplished by selection. The fractions of the selection differentials supposed to represent heritable differences are given in the second part of table 1. The following considerations led to their formulation. LERNER and TAYLOR (1943), using records chiefly from this flock, estimated that the heritability of differences in the production index between families averaging eight daughters was 0.155. This figure is equal to

$$\frac{nr\,G}{1+(n-1)r\,G}$$

where n is the number of sisters in a family (in this case 8), r the genetic correlation between them (in this case 0.5), and G the heritability between individuals (see LUSH 1945). Solving the above equation for G, the heritability of individual records is found to be 0.045. In the present case, the sisters' records of sires and dams were averages of eight and seven pullet records respectively, while the first-year progeny records were averages of 36 pullet records for sires and eight for dams. With these figures as a basis, the respective heritabilities for each of the selection differentials in table 1 can be similarly computed.

The genetic gain which theoretically should have resulted from the selection practiced was then computed as the product of each selection differential and its corresponding estimate of heritability. These figures, given in the third part of table 1, represent the genetic gain expected by selection of the parents at the various stages when selection was practiced. It is noteworthy that early selection was considerably more effective in dams than in sires, whereas the reverse was true of later selection. The first selection of dams was based on both individual performance and completed sisters' records, whereas in sires it was of necessity based only on incomplete sisters' records. This type of selection required two years in the case of the dams and only one in the case of the sires. Progeny-testing was approximately 2.5 times as effective in sires as in dams, the difference being due to the greater accuracy attained by having 4.7 times as many progeny per sire as per dam.

All of the birds in a given year were from sires and dams upon which the first selection had been practiced, while 46 and 32 percent were from progeny-tested sires and dams, respectively. Hence, the average genetic gain of the offspring over the genetic merit of the parental generations may be written as follows

$$\begin{aligned} \text{gain from sires} &= [4.03+0.46\ (11.03)]/2 &&= 4.55 \\ \text{gain from dams} &= [4.46+5.49+0.32\ (4.50)]/2 &&= 5.70 \\ \text{gain from both parents} & &&\overline{10.25} \end{aligned}$$

The gain of 10.25 eggs represents the improvement expected during the period from the hatching of the parents to the hatching of the offspring, which corresponds to the average age of parents. As shown later, the average age of parents was 1.94 years. Thus the ratio $10.25/1.94 = 5.28$ represents the annual gain in production which should have resulted from the selection practiced.

The agreement with the actual gain of 5.6 eggs per year is as close as could reasonably be expected. The heritabilities used in the computations are based on an estimate intended by LERNER and TAYLOR (1943) as a minimum figure and some selection not included in the selection differentials which were used in the computations may have been practiced. This study provides good evidence that a knowledge of selection intensity, heritability and inter-generation span actually enables a breeder to predict the rate of improvement expected from selection (DICKERSON and HAZEL 1944).

EFFECTIVENESS OF PROGENY TESTING

At one time it was considered that the use of progeny-tested parents would

prove an effective tool in promoting genetic improvement which would be applicable to all kinds of livestock and for all economic characters. This belief developed because attention had been centered on the greater accuracy of progeny-test information as compared with that obtained from individual performance or from collateral relatives. Deductive research has indicated more recently that the use of progeny-tested parents may actually result in decreasing the rate of progress as compared with that attainable by earlier selection (DICKERSON and HAZEL 1944). This seems to be particularly true where (1) the length of generation is increased considerably by requiring that the parents be progeny-tested before use, (2) the reproductive rate is high, and (3) the basis for making early selections is relatively accurate. It can be inferred from these conditions that progeny-testing is likely to be more effective for some kinds of animals and for some traits than for others, since these conditions are largely beyond artificial control or manipulation

TABLE 2

Increases in average production of the offspring of part-tested and progeny-tested parents over that from untested parents.

	UNTESTED SIRES		PART-TESTED SIRES		TESTED SIRES		TOTAL	
	NUMBER	EGGS	NUMBER	EGGS	NUMBER	EGGS	NUMBER	EGGS
Untested dams	619	0	344	1.5	118	1.3	1081	0.3
Part-tested dams	191	10.1	132	−2.4	59	11.6	382	6.0
Tested dams	42	9.5	29	11.6	50	22.2	121	8.7
Total	852	1.8	505	1.1	227	7.2	1584	2.3

The data from the present flock afforded an opportunity for investigating experimentally the effectiveness of progeny testing for egg production. Matings were made between untested, partially tested, and fully progeny-tested sires and dams in most of the years. The years 1935, 1937, 1939 and 1941 were again chosen for study. Weighted differences between the progeny from each type of mating and those from untested parents were calculated for each of the four years and combined as shown in table 2. Although the figures appearing therein are not wholly consistent, apparently due to the small numbers of offspring involved, they show that in general the offspring from tested and partially tested parents tended to have higher production than the progeny of untested parents. Thus the progeny from partially tested sires averaged 1.1 eggs and those from partially tested dams 6.0 eggs more than those from untested parents, while those from tested sires and dams, respectively, averaged 7.2 and 8.7 eggs higher. The advantages of the older parents are based on the performance of progeny hatched *after* the parents were promoted to tested ratings, as compared with the performance of progeny hatched in the same years from younger parents which had not yet been tested. These figures are not large in themselves, but when they are compared with the annual rate of gain (5.6 eggs) they show distinct advantages for the progeny of tested parents.

TABLE 3

Average age of parents (in years) for the entire flock and for the families in the upper and lower quartiles of the egg production frequency distribution.

YEAR OF HATCH	POPULATION	SIRES	DAMS	AVERAGE
	Upper quartile	1.42	2.50	1.96
1935	Lower quartile	1.58	2.08	1.83
	Whole flock	1.63	2.51	2.07
	Upper quartile	1.90	2.30	2.10
1936	Lower quartile	1.30	1.80	1.65
	Whole flock	1.53	2.08	1.81
	Upper quartile	1.76	2.41	2.09
1937	Lower quartile	1.71	2.24	1.98
	Whole flock	1.73	2.39	2.06
	Upper quartile	1.27	2.20	1.74
1938	Lower quartile	1.27	2.20	1.74
	Whole flock	1.30	2.20	1.75
	Upper quartile	1.50	2.70	2.10
1939	Lower quartile	1.20	2.40	1.80
	Whole flock	1.52	2.62	2.07
	Upper quartile	1.57	2.14	1.86
1940	Lower quartile	1.29	2.14	1.72
	Whole flock	1.48	2.37	1.93
	Upper quartile	1.46	2.08	1.77
1941	Lower quartile	1.46	2.17	1.82
	Whole flock	1.54	2.21	1.88
	Upper quartile	1.55	2.33	1.94
Average	Lower quartile	1.40	2.15	1.78
	Whole flock	1.53	2.34	1.94

INTERVAL BETWEEN GENERATIONS

The average age of parents is a matter of primary concern in planning breeding programs because it represents the rate at which older generations are being replaced by succeeding ones. Because of the high reproductive capacity of poultry, the interval between generations can be varied considerably depending upon the proportions of old and young parents used for breeding. Table 3 shows the average ages for the sires and dams of the birds hatched from 1935 through 1941. The average ages varied irregularly from year to year, as might be expected where primary emphasis was placed on selecting the apparently superior breeding animals. The figures show no apparent trend either toward younger or older parents as would have been the case if there had been a consistent tendency toward greater use of untested or tested parents. The average age was 1.53 years for sires, 2.34 years for dams, and 1.94 years for both parents.

It may be noted that the sires and dams of the full sister families in the upper quartiles of the frequency distribution of egg production did not average consistently older than those in the lower quartile. Great consistency in this respect is not to be expected, since the heritabilities of the information upon which the parents were selected indicate that many mistakes must have been made in selection. However, the average age of the parents of families in the upper quartile was greater than that of the parents of families in the lower quartile, as might be expected from the conclusion reached concerning the effectiveness of progeny-testing in the preceding section (Table 2). It may also be noted that the parents of families in the lower quartile were consistently younger than the average of all parents.

INTRODUCTION OF BREEDING STOCK

The introduction of breeding stock into herds and flocks of domestic animals corresponds to migration between partially isolated populations. WRIGHT (1940) has shown that partial isolation with limited migration provides a basis for the inter-group selection of genetic systems which is important for the

TABLE 4

Representation of introduced males in the 1941 flock.

YEAR	MALE	REPRESENTATION IN 1941 FLOCK
		Percent
1933	G62	53
1933	G65	96
1934	H90	100
1934	H91	0
1936	K99	5
1938	M88	2
1941	S37	5

evolution of the species as a whole. It should be even more effective in herds and flocks of domestic animals, where the multiplication or elimination of genes from introduced stock can be rigidly controlled by the breeder. LUSH (1946), in summarizing investigations on breeds of domestic animals, found that some degree of subdivision existed in about half of the breeds which have been studied.

Breeding stock was introduced into the production-bred University flock at various times between 1933 and 1941, since which time the flock has been completely closed. The history of the introduced males seemed worth considering in some detail. They are shown in table 4 along with the percentages of the birds in the 1941 flock which trace to them at least once. Males G62 and G65 were half brothers which were first used in 1933. In 1941 53 percent of the birds were descendants of G62 in at least one line, while 96 percent were descendants of G65. The two males imported in 1934 show an even more striking

difference in the extent to which their genes have spread through the flock) H90 being represented in the pedigrees of all of the 1941 flock and H91 in none. Additional introductions of one male each in 1936, 1938 and 1941 were made but these had little effect upon the genetic composition of the present flock. The genes of M88 and S37, which probably were present in low frequency in 1941 (table 4), have since been eliminated entirely, so that they are absent from the 1945 flock, while those of K99 are still present in low frequency. Thus genes from two of the introduced males have been disseminated widely through the flock while those from three others have been eliminated entirely.

Females have been introduced into the production-bred flock from experimental groups on the University farm from time to time. However, it does not seem likely that they could have had much influence in changing the genetic composition of the flock, particularly since they were from the same foundation stock as the original production-bred flock or from families removed from that flock for other studies.

INBREEDING AND RELATIONSHIP

The average coefficients of inbreeding (WRIGHT 1923) are given in table 5 for 1935, 1937, 1939 and 1941, calculated from 1931 as the base year. These coefficients represent the expected average percentage loss or "decay" in heterozygosis since 1931 as a result of gene segregation and subsequent sampling between generations where the effective number of breeding animals is

TABLE 5

Production, inbreeding and relationship data on full-sister families containing five or more birds in relation to the family's position in the flock with respect to egg production.

YEAR OF HATCH	POPULATION	TOTAL NUMBER IN FLOCK	BIRDS INCLUDED		AVERAGE PRODUCTION	AVERAGE INBREEDING COEFFICIENT
			FULL-SISTER FAMILIES NO.	PULLETS NO.		
1935	Upper quartile	485	12	90	241.1	2.0
	Lower quartile		12	87	134.7	2.6
	Whole flock		49	391	190.7	1.5
1937	Upper quartile	592	17	133	224.3	4.1
	Lower quartile		17	126	143.8	3.0
	Whole flock		66	510	185.3	3.4
1939	Upper quartile	465	10	70	238.8	3.8
	Lower quartile		10	82	124.3	9.3
	Whole flock		39	293	184.2	7.0
1941	Upper quartile	638	13	106	269.8	8.9
	Lower quartile		13	98	166.0	8.1
	Whole flock		52	390	219.6	8.1

finite. Changes in heterozygosis which may have occurred due to mutation or selection *per se* are not included in these estimates.

The average inbreeding in 1935 was 1.5 percent, after which it rose to 8.1 percent in 1941. This corresponds to an average increase of about 1.1 percent per year or 2.1 percent per generation. From nine to 14 sires were used in the flock each year, the average number being about 11. The number per generation would be somewhat larger than 11, but would not be more than 17, since about half of the offspring were from sires used more than one year. The number of hens used each year was from about 60 to 90. According to WRIGHT's approximate formula (1931), the rate of inbreeding expected per generation is 100/8M percent in a random mated population consisting of a large number of females and M equally used sires. The actual increase of 2.1 percent per generation corresponds to $M = 100/8\ (2.1) = 5.95$ or approximately 6 as the effective number of sires per generation in the University flock.

Several factors in the mating system tended to make the actual inbreeding differ from that expected on the basis of WRIGHT's formula. Close inbreeding was avoided in most cases by mating birds which had no parents or grandparents in common. Some males and females were introduced after 1931 so that the flock was not strictly closed. These factors would tend to reduce the inbreeding and make the effective number of males larger than the actual number. Family selection was practiced rather intensively (table 1) and required the use of larger numbers of full brothers and sisters as breeders than would have been the case if males had been chosen at random or without reference to the performance of their sibs or parents. In addition, the intensive use of progeny-tested parents made large families of closely related birds available for use as parents. Thus the actual situation in any year was that the males used for breeding consisted of a few sires and their sons (and occasionally grandsons), while many of the females were daughters of the older sires and full sisters of the younger ones. The net result was that the effective number of sires was distinctly smaller than the actual number.

Another aspect of the inbreeding picture is shown by the *inter se* relationship between families. If matings were made at random (except for avoiding full siblings) the inbreeding coefficient would be $R/(2-R)$, where R is the *inter se* relationship between families. The relationships between families in the upper quartile of the egg-production frequency distribution are shown in table 6 for each of four years. The actual inbreeding coefficient is smaller than the coefficient expected on the basis of random mating among non-siblings in each case. This shows that closely related individuals were mated together less frequently than would have occurred under random mating. The two practices followed in the breeding plan, intense family selection and deliberate avoidance of close matings, had opposite effects on the rate of approach to homozygosis, but it is evident that the former was more powerful than the latter.

EFFECT OF INBREEDING ON EGG PRODUCTION

The rate at which homozygosis can be increased without fixing undesirable genes depends on selection intensity, the number of genes involved, and gene

frequency. Since the last two factors are likely to remain unknown for productive characters, the matter can be decided only by experimentation and observation. There are indications (see JULL 1940 for numerous references) that intense inbreeding systems lead to significant deterioration in the productive characters of poultry. In the flock under study, production (figure 1) and inbreeding (table 5) were positively correlated due to a temporal trend. Apparently intense selection can override the deleterious effects of inbreeding when the latter proceeds at the observed rate of 2.1 percent per generation. Nevertheless, it must be kept in mind that the inbreeding is calculable in theoretical

TABLE 6

Inbreeding and relationship in the upper quartile of the flock.

YEAR OF HATCH	*Inter se* RELATIONSHIP	INBREEDING COEFFICIENT	EXPECTED INBREEDING COEFFICIENT
1935	9.2	2.0	5.1
1937	14.0	4.1	8.2
1939	12.7	3.8	7.3
1941	17.4	8.9	10.5

terms only, and that selection of heterozygotes may have retarded slightly the approach to homozygosis indicated by the inbreeding coefficient. Hence it is of interest to determine whether within a given year (where the effect of the time trend would be absent) a relation between production and inbreeding exists. This relation was investigated in three different ways.

Firstly, the inbreeding coefficients for the families in the upper and lower quartiles of the egg-production frequency distribution were computed for the years 1935, 1937, 1939 and 1941 (table 5). The average inbreeding coefficients were larger in the upper than in the lower quartile in two years and smaller in the other two. Secondly, the inbreeding coefficients were calculated for parents of families in these quartiles (table 7). Once more no definite differences were noted. Finally, regression coefficients of egg production on the coefficients of inbreeding were calculated. They were found to be -0.54, -0.46, -5.81 and 0.84 for 1935, 1937, 1939 and 1941, respectively. Since the average for the four years (-1.20) was not statistically significant it cannot be stated with certainty that inbreeding had a detrimental effect on egg production, but at least one of the negative regressions (-5.81) was significant. Hence, the question whether a relatively slow loss of heterozygosis is detrimental to egg production must be left open, but the effect in the concrete case under discussion could not have been large.

RÔLE OF CHANCE

The effect of chance during gene segregation and in the choice of parents is to change gene frequency in a random manner. With N diploid parents (the product of 2N gametes from the preceding generation), the sampling variance of gene frequency at a particular locus is

$$\frac{q\,(1-q)}{2N},$$

q being the frequency of the allele in the generation from which the gametes come. In populations where generations overlap, the rôle of chance is most con-

TABLE 7

Average inbreeding coefficients of the sires and dams of the upper and lower quartiles and of the entire flock by years.

YEAR OF HATCH OF FLOCK	PULLETS IN UPPER QUARTILE		PULLETS IN LOWER QUARTILE		ALL PULLETS	
	SIRES	DAMS	SIRES	DAMS	SIRES	DAMS
1935	0	1.0	0	0	0	0.3
1937	0.2	2.9	0.4	2.8	0.6	1.6
1939	5.9	1.6	8.1	1.7	6.3	2.7
1941	8.6	4.6	7.3	6.4	8.2	5.2

veniently measured by the inbreeding coefficient. Thus the standard error per generation in the present flock is $100\sqrt{.021\ q\,(1-q)} = 15\sqrt{q\,(1-q)}$ percent. This is about 7.5 percent for genes with a frequency near 50 percent and 1.5 percent for genes with a frequency near one percent.

As an example of the importance of chance, we may consider the gene frequency array expected for 100 loci with an original frequency of 50 percent after one generation of segregation. The expected array is:

Frequency (percent)	Number of loci
Below 35	2.1
35 –42.5	13.6
42.5–50	34.2
50 –57.5	34.2
57.5–65	13.6
Above 65	2.1

When gene frequency is near 0 or 100 percent, chance operates with greater finality, although with less magnitude quantitatively than when frequency is in the intermediate range. Thus genes having a frequency of less than 5 percent would appear to be in danger of extinction each generation. Of 100 genes with low frequency, it is to be expected that a few would be lost entirely from the flock, most would be transmitted without great change in frequency, while a few (which happened to be present in some of the males chosen as sires) would appear in the succeeding generation in greatly increased frequency. Since sampling accidents occur in a random manner they may or may not be cumulative.

Hence, the genetic constitution of a flock no larger than this is likely to be altered considerably in a few generations by the effects of chance alone.

DISCUSSION

The actual improvement in egg production in the 12 years covered by this study was from an average of about 120 eggs to about 220 eggs per bird per year. This does not necessarily represent the maximum possible rate of progress, but is sufficient to attest to the power of the genetic forces brought to bear on the flock.

In general, breeding programs may be designed to emphasize the rôles of selection, of migration, or of chance, individually, so that only one force is responsible for changes in the genetic composition of the population (except that mutation cannot be controlled). Deductive studies (summarized by WRIGHT 1942) indicate that most favorable conditions for genetic advance are attained when the evolutionary forces are balanced against each other rather than when any one completely dominates the situation. The breeding program used in this flock seems to be well balanced from this standpoint, although undoubtedly some refinements might make it more effective. Its chief criticism may be that selection has been so intense that rare beneficial genes have been eliminated by chance or that certain beneficial combinations of genes have been prevented from occurring. All of the selection has been in one direction and for one kind of genetic system, whereas, if control by selection had been somewhat less rigid, a greater number of gene combinations and genetic systems would have been formed and tested. The specific effects of chance, migration and selection, together with interrelations between them, may be next considered in that order.

The rôle of chance in determining changes in gene frequency from one generation to another has been quite important in this flock. This was in part a consequence of the kind of selection practiced. LUSH (1946) pointed out that intense selection was accompanied by restriction of ancestry in small populations of domestic animals. Although close mating was avoided where possible in this flock, inbreeding rose more rapidly than would have been expected in a flock of equal size where the parents were chosen at random. The intense family selection was responsible for the additional inbreeding and for the corresponding increase in the importance of random changes in gene frequency.

It seems probable that selection has been sufficiently intense to avoid the random fixation of genes which had undesirable effects unless their initial frequency was very high. The evidence concerning the detrimental effect of inbreeding was inconclusive but the effect was not pronounced if it existed at all. LUSH (1945, p. 284) stated "It is unlikely that inbreeding rates as high as three or four percent (per generation) can go on forever without harm, but certainly they can be continued for many generations." Nothing can be added to this statement from the present study except that an appreciable rate of improvement was maintained for several generations while the rate of inbreeding was more than two percent per generation.

Migration, or the introduction of breeding stock, was not an important fac-

tor quantitatively, since only seven sires have been introduced among approximately 100 used. Family selection, when combined with migration, provides an effective means of testing new gene combinations and accepting or rejecting them more quickly than is likely to occur in nature. In this case, the intense family selection practiced quickly eliminated genes from three of these sires and carried the frequency of genes from two others to relatively high levels.

Selection was the only directive force utilized in the breeding program. Considerable significance is attached to the general agreement between the actual rate of improvement and that calculated on the basis of the selection which has been practiced. Since this seems to be the first experimental test of the selection principles deduced in theoretical studies, the agreement between actual improvement and expected improvement was sufficiently close to be gratifying. To some extent then, those principles can be employed in applied breeding programs with greater confidence in their validity.

The three factors that determine the effectiveness of selection are selection intensity, selection accuracy or heritability, and the average age of parents. Although they can be manipulated by the breeder, they are not independent of each other. Thus accuracy of selection and the average age of parents exercise a pull in opposite directions. Similarly, as shown in table 1, the selection bases with lower heritabilities permit higher selection differentials than those with higher heritabilities. Hence, there undoubtedly must exist an optimum combination of these forces to provide the maximum rate of improvement. Whether or not the optimum combination was attained in the present flock is not an important question in this discussion. The significant point is that the rate of progress predictable from the factors discussed was realized, thereby substantiating the statistical and genetic principles upon which they were based.

SUMMARY

The rôles of selection, chance and migration were analyzed with respect to the improvement in egg production in the UNIVERSITY OF CALIFORNIA poultry flock over a period of 12 years. The important features of the mating system used were: annual flock size from 400–700 pullets, semi-closed system of breeding, emphasis on family and progeny performance, selection and breeding from females two years of age or older, and deliberate avoidance of close mating.

The analysis indicated that the theoretical gains expected on the basis of known selection intensity, heritability and average interval between generations were in close agreement with the actual gains realized (5.28 vs. 5.6 eggs per year). This is interpreted as a verification of the principles of population genetics derived deductively by earlier workers.

Progeny-testing was found to have made a significant contribution to the improvement obtained in spite of the increase in the average age of parents incident to its use.

The rate of approach to homozygosis was found to be of the order of two percent per generation, or one percent per year, in spite of the mating system used. This situation arose as a result of family selection which leads to a reduction

from 11–17 actual sires per generation to a calculated number of six effective sires. No significant detrimental effects of this rate of inbreeding on egg production could be established.

The rôle played by chance in changing gene frequencies was found to be rather large, the standard error of chance segregation being between 7.5 percent for genes with a frequency of 50 percent and 1.5 percent for genes with frequencies near one percent.

Migration was not an important factor quantitatively in this population, since the number of introduced sires was relatively low. It was shown that artificial selection provides potent means for rapid incorporation or elimination of introduced genes.

As a general conclusion, it may be stated that the currently accepted principles of population genetics (excluding the rôle of mutation) are empirically verifiable and lead to predictable rates of improvement in populations subjected to artificial selection.

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THE EIGHTH INTERNATIONAL CONGRESS OF GENETICS will be held in Stockholm in the summer of 1948. The date will be announced later, when more is known about other congresses planned for 1948. Swedish geneticists have formed an Organization Committee for the Congress, with Professor Gunnar Dahlberg, of the University of Uppsala, as chairman, and Professor Gert Bonnier, Institutet för Husdjursförädling, Wiad, Eldtomta, as general secretary.

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